SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD

Dissertation submitted to

The Tamil Nadu Dr.M.G.R Medical University

Chennai-600032.

In partial fulfilment of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

PUSHPARAJ. M

(Reg.No: 261330803)

Under the guidance of

Dr.M. SENTHILKUMAR. M.Pharm., Ph.D

Vice Principal& Head

Department of Pharmaceutical Analysis

Cherraan's College of Pharmacy

OCTOBER 2017

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

CHERRAAN'S COLLEGE OF PHARMACY

COIMBATORE– 39.

CERTIFICATE

CERTIFICATE

This is to certify that this dissertation entitle **"SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD"** Submitted by **Reg.no:261330803** to the Tamil Nadu Dr.M.G.R Medical University, Chennai in partial fulfilment for the degree of **MASTER OF PHARMACY IN PHARMACEUTICAL ANALYSIS** is a bonafied work carried out under the guidance and supervision of **Dr. M. SENTHILKUMAR. M.Pharm., Ph.D** Vice Principal & Head, Department of Pharmaceutical Analysis, Cherraan's College of Pharmacy Coimbatore-39.

I have fully satisfied with his performance and work. I have forward this dissertation work for evaluation.

Station:

Date:

Dr. N. THIRUMOORTHY, M.Pharm., Ph.D.,
Principal
Cherraan's College of Pharmacy

CERTIFICATE

This is to certify that this dissertation entitled **"SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD"** Submitted by **Reg.no:261330803** to the Tamil Nadu Dr.M.G.R Medical University, Chennai in partial fulfilment for the degree of **MASTER OF PHARMACY IN PHARMACEUTICAL ANALYSIS** is a bonafied work carried out by the candidate under my guidance and supervision in the Department of Pharmaceutical Analysis, Cherraan's College of Pharmacy Coimbatore-39.

I have fully satisfied with his performance and work. I have forward this dissertation work for evaluation.

Station:

Date :

Dr.M. SENTHILKUMAR. M.Pharm.,Ph.D

Vice Principal & Head
Department of Pharmaceutical Analysis

CERTIFICATE

This is to certify that this dissertation entitled **"SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD"** submitted by **Mr. PUSHPARAJ. M** to the Tamil Nadu Dr.M.G.R Medical university, Chennai in partial fulfilment for the degree of **MASTER OF PHARMACY IN PHARMACEUTICAL ANALYSIS** is a bonafied work carried out by the candidate under my guidance at **BANNER PHARMACAPS, BANGALORE** during the academic year 2016-2017.

Station:
Date :

Mr. RS Parihar
Co-guide

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD"** submitted by,Reg.No**:261330803** to The Tamil Nadu Dr. M. G. R Medical University, Chennai in partial fulfilment for the Degree of **MASTER OF PHARMACY IN PHARMACEUTICAL ANALYSIS** is a bonafied work carried out during the academic year 2016-2017 by the candidate at Department of Pharmaceutical Analysis, Cheerran's College of Pharmacy, Coimbatore and was evaluated by us.

Examination centre:

Date:

Internal Examiner

External Examiner

DECLARATION

I hereby declare that this dissertation entitled **"SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD"** submitted by me, in partial fulfilment of the requirements for the degree of **MASTER OF PHARMACY IN PHARMACEUTICAL ANALYSIS** to The Tamil Nadu Dr.M.G.R Medical university, Chennai is the result of my original and independent research work carried out under the guidance of **Dr. M. SENTHILKUMAR, M.Pharm.,Ph.D.,** Vice Principal & Head Department of Pharmaceutical Analysis, Cherraan's College of Pharmacy, Coimbatore-39, & Co-Guide **Mr. Rajendra Singh Parihar, BANNER PHARMACAPS, BANGALORE** during the academic year 2016- 2017.

Station:

Date: (Reg.No.261330803)

ACKNOWLEDGEMENT

First of all I would like to thank god for his blessings to do this research work successfully. With immense pleasure and pride, I would like to take this opportunity in expressing my deep sense of gratitude to my beloved guide **Dr M. SenthilkumarM.Pharm., Ph.D** Vice principal & Head, Department of Pharmaceutical Analysis, Cherraan's College of Pharmacy under whose active guidance, innovative ideas, constant inspiration and encouragement of the work entitled "**SIMULTANEOUS ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE BY RP-HPLC METHOD**" has been carried out.

I wish to express my deep sense of gratitude to honorable **K.C. Palanisamy,** Chairman of Cherraan's group of Institutions for the facilities provided to me in this institution.

My sincere thanks to our respected and beloved Principal **Dr. N.Thirumoorthy,M.Pharm.,Ph.D.,**Principal, Cherraan's College of Pharmacy for his encouragement and also providing all facilities in this institution to the fullest possible extent enabling me to complete this work successfully.

PUSHPARAJ. M

(261330803)

DEDICATED TO MY BELOVED PARENTS, TEACHERS, FRIENDS AND ALMIGHTY

ABBREVIATIONS

- JP: Japanese Pharmacopoeia
- ETO : Etoricoxib
- SER : Serratiopeptidase
- CNS: Central Nervous System
- NSAIDs: Non-Steroidal Anti Inflammatory Drugs
- COX: Cyclo-oxygenase
- c-GMP: cyclic Guanosine monophosphate
- DMSO: Di-Methyl Sulfoxide
- FDA : Food and Drug Administration
- ICH : International Conference of Harmonization
- USP : United States Pharmacopoeia
- API: Active Pharmaceutical Ingredient
- UV: Ultraviolet
- RP-HPLC: Reverse Phase High Performance Liquid Chromatography
- HPTLC: High Performance Thin Layer Chromatography
- NMR: Nuclear Magnetic Resonance
- LC-MS: Liquid Chromatography-Mass Spectroscopy
- GC-MS: Gas Chromatography-Mass Spectroscopy
- ESI: Electro Spray Ionization
- TOF: Time of Flight
- LC-MS-MS: Liquid Chromatography-Tandem Mass Spectroscopy
- UPLC: Ultra Performance Liquid Chromatography
- ACN: Acetonitrile
- OPA: Ortho Phosphoric acid
- S.D.: Standard Deviation
- RSD: Relative Standard Deviation
- CV: Coefficient of Variance
- S/N: Signal to Noise Ratio

- Rf: Retardation Factor
- i.d.: Internal Diameter
- µg: Microgram
- mg : Milligram
- ml: Milliliter
- µl: Microliter
- mm: Millimeter
- cm: Centimeter
- ng: Nanogram
- MCC : Microcrystalline Cellulose
- Min.: Minutes
- AUC: Area Under Curve
- %: Percentage
- AR: Analytical Grade Reagent
- °C: Degree Centigrade
- M: Molar
- r^2: Correlation Coefficient
- Rt : Retention Time
- v/v : Volume/Volume
- Std.: Standard
- LOD: Limit of Detection
- LOQ: Limit of Quantification
- PDA: Photo Diode Array
- ANOVA: Analysis of Variance
- C.F.: Correction Factor
- d.f.: Degree of Freedom
- C.S.S.: Column Sum of Squares
- T.S.S.: Total Sum of Squares

CONTENT

CONTENT

List of Tables

List of Figures

INTRODUCTION

1. INTRODUCTION

1.1 INTRODUCTION OF MUSCLE SPASM[1-3]:

Muscle spasm: A muscle spasm, or muscle cramp, is an involuntary contraction of amuscle. Muscle spasms occur suddenly, usually resolve quickly, and are often painful. Muscles are complex structures that cause movement in the body. There are three types of muscle in the body:

- Heart muscle pumps blood (cardiac muscle).
- Skeletal muscle moves the external body parts, like the arms and legs, and the neck.
- Smooth muscle moves portions of hollow structures inside the body. Examples include the muscles that line the stomach and intestine, muscles that line large arteries and the muscles of the uterus.

Causes:

There are a variety of causes of muscle spasms.

- Muscle is overused and tired, particularly if it is overstretched
- Unfamiliar exercise activities
- Atherosclerosis
- Systemic illnesses like diabetes, anemia and kidney and thyroid disease

Symptoms and signs:

The symptoms of muscle spasm depend upon the muscle involved and the circumstances leading up to the spasm. Skeletal muscle spasm usually involves muscles that are being asked to do excessive work. There is acute onset of pain as the muscle contracts. Most often, the spasm resolves spontaneously after a few seconds though it may last many minutes or longer. Usually, the patient will feel the need to stretch the muscle involved, thus relieving the spasm and resolving the episode. Smooth muscle spasm will cause colicky pain that comes and goes. The symptoms will depend upon the organ involved.

Treatment of muscle spasm:

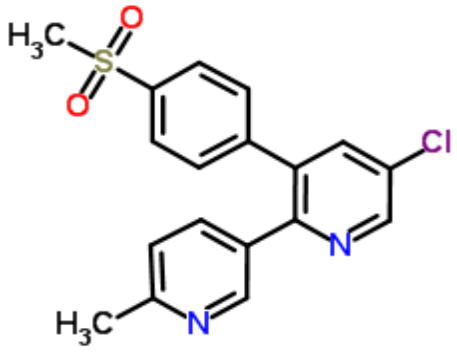
The treatment of smooth muscle spasm also depends upon the underlying cause. Various drugs used for muscle spasms are shown in table 1.1,

Table 1.1: Classification of drugs

Peripherally acting Muscle relaxants	Non-depolarizing agent	Curare alkaloids	Tubocurarine, Dimethyltubocurarine
		4-Ammonium agents	Atracurium, Cisatracurium,Gallamine
	Depolarizing agent	Choline derivatives	Succinylcholine,
	Ach release inhibitors	Botalinum toxin	
Centrally acting Muscle relaxants	Carbamic esters	Meprobamate, Methocarbamol, Tybamate	
	Benzodiazepines	Diazepam, Lorazepam, Nitrazepam	
	Anticholinergics	Orphenadrine	
	Piperidine derivatives	Tolperisone,Etoricoxib	
	Others	Quinine, Baclofen, Thiocolchicoside	
Directly acting Muscle relaxants	Dantrolene		
Analgesic drugs	Diclofenac, Ibuprofen, Serratiopeptidase		

1.2 DRUG PROFILE OF ETORICOXIB[4-5]:

Table 1.2: Drug Profile of ETORICOXIB

Generic name	Etoricoxib
Description	Etoricoxib is a white to off-white powder
Chemical structure	
Chemical name	5-chloro-6'-methyl-3-[4- (methylsulfonyl)phenyl]-2,3'-bipyridine
Formula	$C_{18}H_{15}ClN_2O_2S$
Molecular mass	358.84.
Category	Analgesic
Solubility	Very soluble in acetic acid, methanol, freely soluble in water and in ethanol, soluble in acetic anhydride, slightly soluble in acetone, and practically insoluble in diethyl ether
Mechanism of action	This NSAID is a selective COX2 inhibitor (blocking the action of a substance in the body called cyclo-oxygenase. Cyclo-oxygenase is involved in producing prostaglandins, in response to injury or certain diseases. These prostaglandins cause pain, swelling and inflammation). This inhibition results the analgesics and anti-inflammatory action peripherally

1.3 DRUG PROFILE OF SERRATIOPEPTIDASE[6-7]:

Table 1.3: Drug Profile of Serratiopeptidase

Generic Name	Serratiopeptidase
Description	Off-white to light brown coloured powder, having characteristics odour
Molecular mass	45,000 – 60,000 Da
Category	Anti-inflammatory
Mechanism of action	The mechanism of action of Serratiopeptidase appears to be hydrolysis of histamine, bradykinin and serotonin. Serratiopeptidase also has a proteolytic and fibrinolytic effect. This is achieved by dissolving the complement (specific proteins responsible for inflammation) and increasing the plasmin activity by inhibiting the plasmin inactivators.

1.4 INTRODUCTION TO VALIDATION OF ANALYTICAL METHODS (USP/ICH) [8]

Method validation, according to the United States Pharmacopoeia (USP), is performed to ensure that an analytical methodology is accurate, specific, reproducible, and rugged over the specified range that an analyte will be analyzed. Regulated laboratories must perform method validation in order to be in compliance with FDA regulations. In a 1987 guideline (Guideline for submitting samples and analytical data for methods validation), the FDA designated the specifications in the current edition of the USP as those legally recognized when determining compliance with t he Federal Food, Drug and Cosmetic Act. It can be referred to as the “eight steps of method validation,” as shown in Figure 1.1.

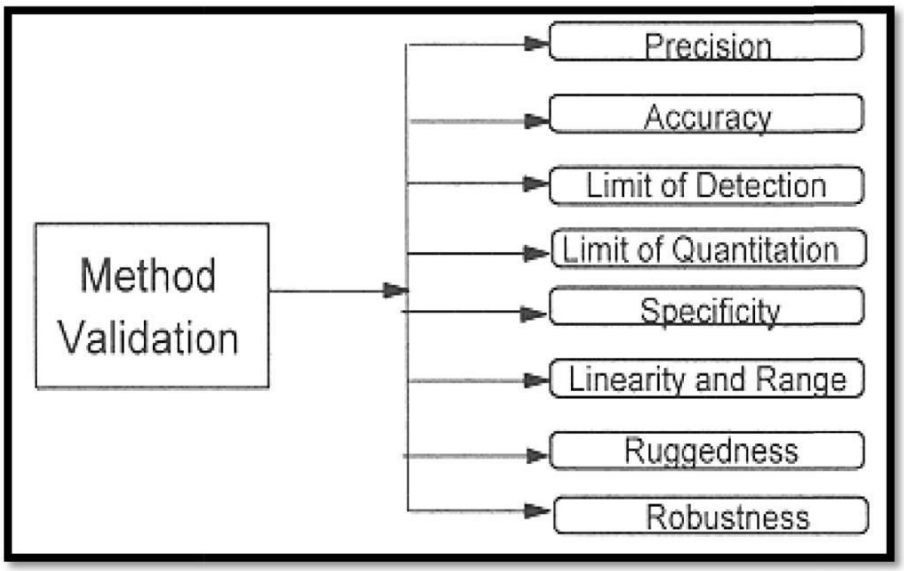


Figure 1.1: Eight steps of method validation

ICH has suggested the guideline for validation of analytical procedure under the heading “Validation of Analytical Procedures: Methodology” in the section Q2B. ICH divided the validation characteristics somewhat differently, as outlined in Figure 1.2.

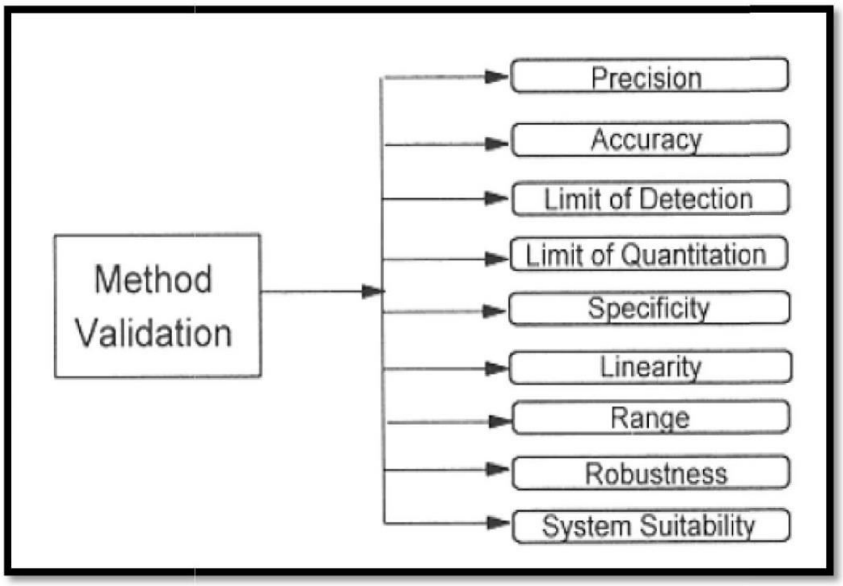


Figure 1.2: ICH method validation parameters

The differences between the USP and ICH terminology is, for the most part, one of semantics—with one notable exception. ICH treats system suitability as a part of method validation, whereas the U SP treats it in a separate chapter.

1.4.1 Accuracy

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is measured as the percentage of analyte recovered by assay, by spiking samples in a blind study.

1.4.2 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the %RSD for a statistically significant number of samples.

According to the ICH, precision should be performed at three different levels: repeatability, intermediate precision, and reproducibility.

(a) Repeatability refers to the results of the method operating over a short time interval under the same conditions (inter-assay precision). It should be determinedfromaminimum of nine determinations covering the specified

(b) range of the procedure or from a minimum of six determinations at 100% of the test or target concentration.

(b) Intermediate precision refers to the results from within-lab variations due to random events such as differences in experimental periods, analysts, equipment, and so forth. In determining intermediate precision, experimental design should be analyzed so that the effects of the individual variables can be monitored.

(c) Reproducibility refers to the results of collaborative studies among laboratories.

(d) Recommended data: Precision studies should include the S.D., R.S.D., C.V. and confidence interval.

1.4.3 Specificity

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix.

1.4.4 Limit of detection

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated. Several approaches for the determining the detection of limit is possible, depending upon weather the procedure is an instrumental or non-instrumental.

1.4.5 Limit of quantification

The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Several approaches for the determining the quantification limit is possible, depending upon weather the procedure is an instrumental or non-instrumental.

1.4.6 Linearity

The linearity is the ability of analytical procedure to produce test results which are proportional to the concentration of analyte in samples within a given concentration range, either directly or by means of a well-defined mathematical transformation. Linearity should be determined by using a minimum of six standards whose concentration span 80–120% of the expected concentration range.

1.4.7 Range

Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method. The range is normally expressed in the same units as the test results obtained by the method.

1.4.8 Ruggedness

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under variety of conditions, expressed as % RSD. These conditions include differences in laboratories, analysts, instruments, reagents, and experimental periods.

1.4.9 Robustness

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength, or temperature, and determining the effect on the results of the method. As documented in the ICH guidelines, robustness should be considered early in the development of a method.

1.4.10 System suitability

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD retention time and area for repetitive injections) are determined and compared against the specifications set for the method.

Table 1.4: System suitability parameters and recommendations

Parameters	Recommendation
Capacity factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferences (impurity, excipients, degradation product, internal standard, etc.
Tailing factor (T)	T of </= 2
Theoretical plates (N)	In general should be > 2000

1.5. INTRODUCTION TO SPECTROPHOTOMETRIC METHODS [9]

Various spectrophotometric methods are available for the analysis of drugs in combined dosage forms, like

- Simultaneous equations method
- First order derivative method
- Area under curve method

1.5.1. Simultaneous equations method

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λmax of the other (as shown in figure λ1and λ2), it may be possible to determine both drugs by the technique of simultaneous equation. In this method, the absorbances of the solutions are measured at the λ max of both the drugs. The criteria are that the ratios [(A2/A1) / (ax2/ax1)] and [(ay2/ay1) / (A2/A1)] should lie outside the range 0.1-2.0.

Concentration of both drugs is calculated by solving the simultaneous equations 1 & 2.

$$Cx = (A2\ aY1 - A1\ aY2) / (aY1\ aX2 - aY2\ aX1) \text{ ----- (1)}$$

$$Cy = (A1\ aX2 - A2\ aX1) / (aY1\ aX2 - aY2\ aX1) \text{ ------ (2)}$$

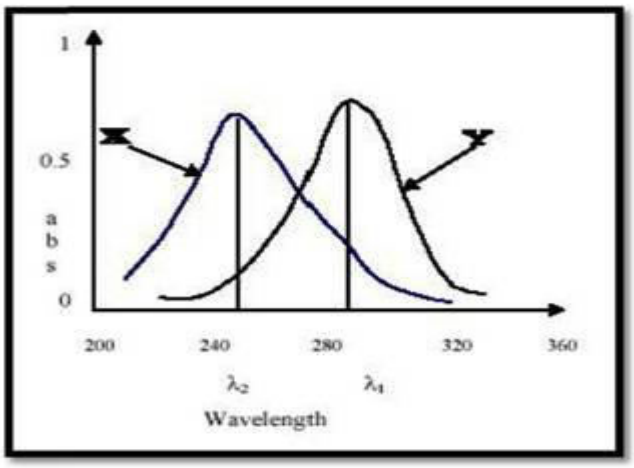


Figure 1.3: Overlain U.V. absorption spectra of two drugs

Where, A1, A2= Absorbances of mixture at λ1 & λ2 respectively, ax1 =Absorpitivity of first drug at λ1, ax2 = Absorpitivity of first drug at λ2, ay1=Absorpitivit y of second drug at λ1, ay2 = Absorpitivity of second drug at λ2.

1.5.2 Derivative Spectrophotometric Method:

For the purpose of spectral analysis in order to relate chemical structure to electronic transitions, and for analytical situations in which mixture contribute interfering absorption, a method of manipulating the spectral data is called derivative spectroscopy.

Derivative spectrophotometry involves conversion of a normal spectrum to its first, second and higher derivative spectrum. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, zero order, or D^0 spectrum.

The first derivative spectrum is a plot of the rate of change of absorbance with wavelength against wavelength i.e. a plot of the slope of the fundamental spectrum against wavelength or a plot. The maximum positive and maximum negative slope respectively in the D spectrum correspond with a maximu m and minimum

respectively in the D^1 spectrum. The □max in D spectrum is a wavelength of zero slope and gives dA/d ƛ = 0 in the D^1 spectrum.

The second derivative D^2 spectrum is a plot of the curvature of the D spectrum against wavelength or a plot o f $d^2A/d\lambda^2$ vs. λ. The maximum negative curvature in the D spectrum gives two small maxima called satellite bands in the D^2 spectrum, and the maximum positive curvature in the D spectrum gives two small maxim a called satellite bands in the D^2 spectrum. The wavelength of maximum slope and zero curvature in the D spectrum correspond with cross-over points in the D^2 spectrum.

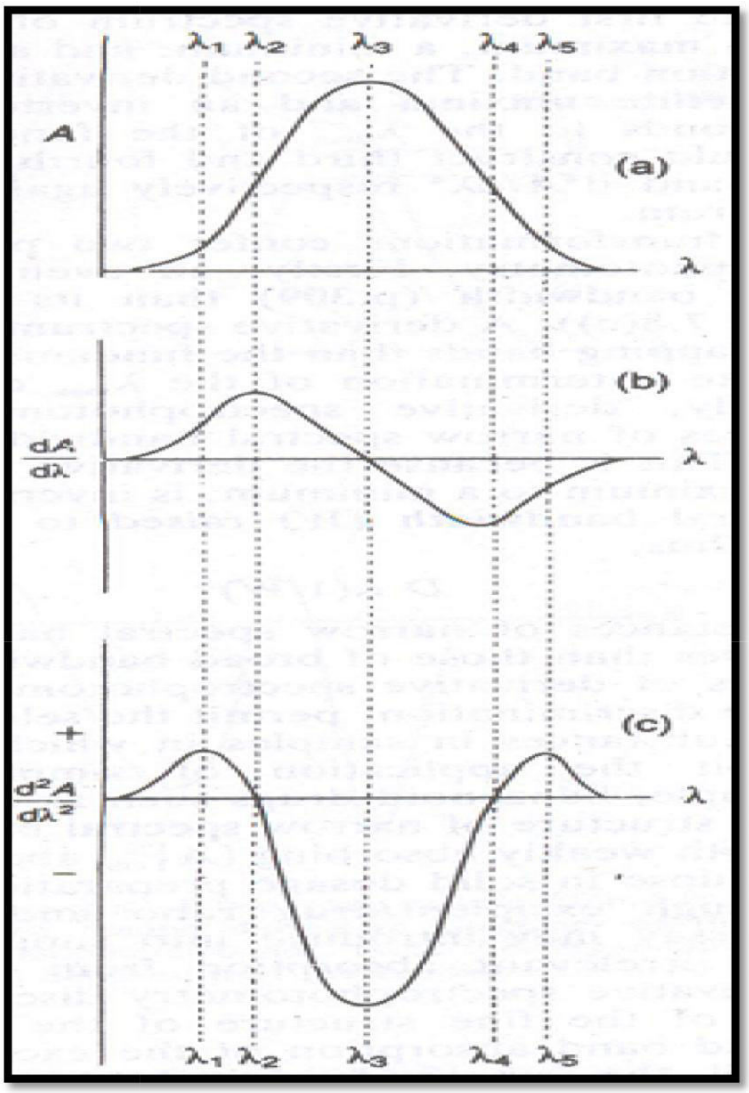


Figure 1.4: (a) The Zeroth, (b) first, (c) second derivative spectra of guassian band

These spectral transformations confer two principal advantages on derivative spectrophotometry. Firstly, Zero order spectrum is of narrower spectral bandwidth than its fundamental spectrum. A derivative spectrum therefore shows better resolution of overlapping bands than the fundamental spectrum and may permit the accurate determination of the ƛmax of the individual bands. Secondly, derivative spectrophotometry discriminates in favour of substances of narrow spectral bandwidth against broad bandwidth substances. All the amplitudes in the derivative spectrum are proportional to the concentration of the analyte, provided that Beer's law is obeyed by the fundamental spectrum.

The enhanced resolution and bandwidth discrimination increases with increasing derivative order. However, it is also found that the concomitant increase in electronic noise inherent in the generation of the higher order spectra, and the consequent reduction of the signal to noise ratio, place serious practical limitations on the higher order spectra. For quantitative purposes, second and fourth derivative spectra are the most frequently employed derivative orders.

A first-order derivative is the rate of change of absorbance with respect to wavelength. A first order derivative starts and finishes at zero. It also passes through zero at the same wavelength as maximum of the absorbance band. Either sides of this point are positive and negative bands with maximum and minimum at the same wavelengths as the inflection points in the absorbance band. This bipolar function is characteristic of all odd-order derivatives.

Derivative spectra may be generated by any of three techniques. The earliest derivative spectra were obtained by modification of the optical system. Spectrophotometers with dual monochromator set a small wavelength interval (typically 1-3 nm) apart or with the facility to oscillate the wavelength over a small range, are required. In either case the photodetectror generates a signal with amplitude proportional to the slope of the spectrum over the wavelength interval. Instruments of this type are expensive and are essentially restricted to the recording of first derivative spectra only.

The second technique to generate derivative spectra is electronic differentiation of the spectrophotometer analog signal. Resistance Capacitance modules may

be incorporatedin series between the spectrophotometer and recorder to provide differentiation of the absorbance, not with respect to wavelength, but with respect to time, thereby producing the signal dA/dt. If the wavelength scan rate is constant (d/dt = Ce), the derivative with respect to wavelength is given by,

$$DA/d\lambda = (dA/dt)/(d/dt) = (dA/dt)(1/C)$$

Derivative spectra obtained by RC method are highly dependent on instrumental parameters, in particular the scan speed and the time constant. It is essential, therefore, to employ a standard solution of the analyte to calibrate the measured value the instrumental conditions selected.

The third technique is based upon microcomputer differentiation. Microcomputers incorporated into or interfaced with the spectrophotometer may be programmed to provide derivative spectra during or after the scan, to measure derivative amplitudes between specified wavelengths and to calculate concentrations and associated statistics from the measured amplitude.

1.5.3 Area under curve method

This method utilizes two wavelength ranges. From overlain spectra of both drugs the area under curve is determined at both the selected analytical wavelength ranges. Within the above selected wavelength ranges, the area under curve was determined for both drugs.

In this method the concentration of both drugs is calculated by solving the equations 1 & 2.

$Cx = (A2\ aY1 - A1\ aY2) / (aY1\ aX2 - aY2\ aX1)$ ----- (1)

$Cy = (A1\ aX2 - A2\ aX1) / (aY1\ aX2 - aY2\ aX1)$ ------ (2)

Where, Cx = Concentrations of drug X,

Cy = Concentrations of drug Y,

A1 = Area at first wavelengthrange,

A2 = Area at second wavelength range,

aX1 and aY1 are AUC constants of X and Y respectively at first wavelength range, aX2 and aY2 are AUC constants of X and Y respectively at second wavelength range,

AUC constant = Area/ concentration in gm/l.

1.6 INTRODUCTION TO HPLC METHODS[10-12]

Most of the drugs in multi component dosage forms can be analyzed by HPLC method because of the several advantages like:

- Speed
- Greater sensitivity
- Improved resolution
- Reusable columns
- Easy sample recovery, handling and maintenance
- Instrumentation tends itself to automation and quantification
- Precise and reproducible results
- Calculations are d one by integrator itself.

Different modes of separation in HPLC are available like, normal phase mode and reversed phase mode. In the normal phase mode, the stationary phase is polar and the mobile phase is non-polar in nature. In this technique, non-polar compounds travel faster and are eluted first. This is because of the lower affinity between the non-polar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most of the drug molecules are polar in nature and hence take longer time to elute.

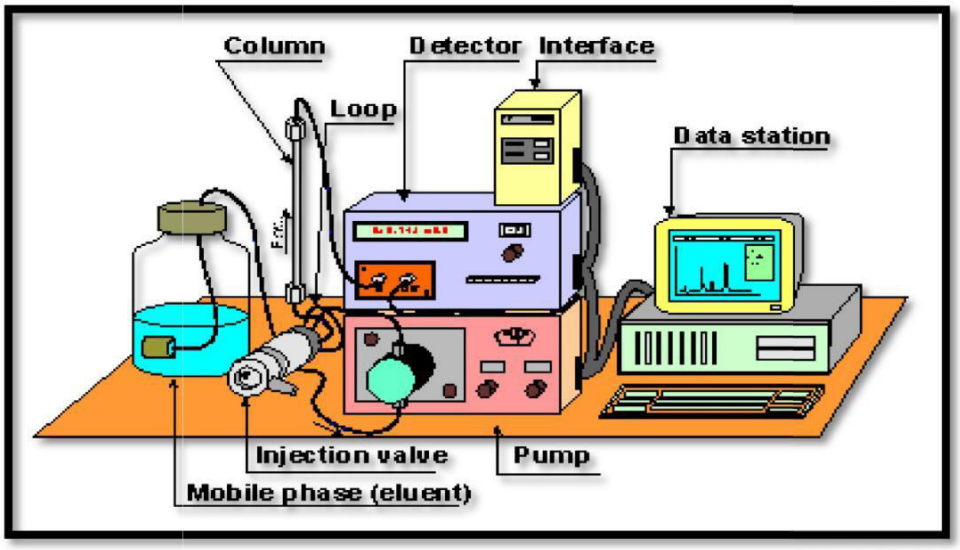


Figure 1.5: Schematic diagram of HPLC instrument

Various components of HPLC are:

- A solvent delivery system, including pump
- Sample injection system
- A chromatographic column
- A detector
- A strip chart recorder
- Data handling device and microprocessor control.

Solvent delivery system

A mobile phase is pumped under pressure from one or several reservoir and flows through the column at a constant rate. For normal phase separation eluting power increases with increasing polarity of the solvent but for reversed phase separation, eluting power decreases with increasing polarity.

Pumps:

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity.

Three main types of pumps are used in the HPLC system.

1. Displacement pump
2. Reciprocating pump
3. Pneumatic or constant pressure pump

b) Sample injection system

There are three important ways of introducing the sample into injection port.

1. Loop injection

2.Valve injection

3.On column

(c) Chromatographic column

The column is usually made up of heavy glass or stainless steel tubing to withstand high pressure. The columns are usually 10-30 cm long and 4-10 mm inside diameter containing stationary phase at particle diameter of 25 μm or less.

Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

Column packing:

The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC.

1. Porous, polymeric beds
2. Porous layer beds
3. Totally Porous silica particles (dia. <10 μm)

d) Detectors

The function of the detector in HPLC is to monitor the mobile phase as it merges from the column. Detectors are usually of two types:

1. Bulk property detectors: It compares overall changes in a physical property of the mobile phase with and without an eluting solute. e.g. refractive index, dielectric constant or density.

2.Solute property detectors: It responds to a physical property of the solute which is not exhibited by the pure mobile phase. e.g. UV absorbance, fluorescence or diffusion current. Such detectors are about 1000 times more sensitive giving a detectable signal for a few nanogram (ng) of sample.

LITERATURE REVIEW

2. REVIEW OF LITERATURE

Gangane PS et al[13] investigated and studied a HPLC method for the estimation of etoricoxib in both tablet and bulk formulations by using LC-10 ATVP Shimadzu Liquid Chromatography with Hyper ODS 2 C18 size 4.5×250 mm column as a stationary phase.Using methanol as solvent of flow rate of 1ml/minute. Both interday and intraday precision was found to be within the limits. accuracy and reproducibility were found to be satisfactory.

Adidala RR et al[14]examined simultaneous estimation of paracetamol, aceclofenac and serratiopeptidase in tablet dosage form using C18 column (250mm×4.6, 5µ) with mobile phase consisting of acetonitrle water and glacial acetic acid with a flow rate of 1.0ml/minute. Precision of drugs was observed to be less than 2.0 of %RSD by mean of 6 determinations. Linearity was observed over the concentration range 1–50 µg/ml (r2=0.998) with regression equation y = 36941x – 61362 for Paracetamol, Aceclofenac 1–50 µg/mL (r2=0.997) with regression equation y = 42784x + 23799 and Serratiopeptidase 1-50 µg/mL (r2= 0.998) with regression equation y = 1904x + 22854. LOD and LOQ of Paracetamol, aceclofenac and serratiopeptidase were found to be 2.27µg/ml, 1.1µg/ml, 3.62µ/ml and 6.88µg/ml, 3.33µ/ml, 10.9µg/ml respectively. The method was validated as per ICH guidelines.

Syed Mujdaba A et al[15] developed a novel, simple, sensitive and rapid Chromatographic RP-HPLC method for simultaneous estimation of NSAIDS (Serratiopeptidase and Diclofenac sodium) from pharmaceutical formulation. The present isocratic method was carried out on analytical column- WATERS XTERRA RP8 (4.6x150, 5 microns) with pH -3 adjusted mobile phase [Ortho Phosphoric acid buffer: methanol 70:30 (v/v)] at the flow rate 1.0 mL/min. The detection was carried out at wave length (λ max) 262 nm. The average retention time of diclofenac sodium was 3.763 min and Serratiopeptidase was 5.480 min. They developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation and solution stability.

Asihsh Sharma et al[16] designed a simple, rapid, economical, precise and accurate UV spectrophotometric method for simultaneous estimation of Nimesulide and Serratiopeptidase in bulk drug and combined dosage form (Tablet Dosage form) has been developed. The method employed solving of simultaneous equation based on measurement of absorbance at two wavelengths, 276.8 nm and 389.2 nm, λmax of Serratiopeptidase and Nimesulide respectively. The % recovery for Serratiopeptidase and Nimesuldie was found to be 99.3% to 101.1% and 99.76 % to 100.22%.

Ashok R Parimar et al[17] developed UV- visible Spectrophotometric and validated for Simultaneous estimation of Aceclofenac and Serratiopeptidase in tablet dosage form using double beam UV Spectrophotometer of thermo Electron Corporation (Heλios α) with (Ethanol + Water) as a solvent. Absorption maxima of Aceclofenac and Serratiopeptidase in Ethanol diluted with water was found to be 316 nm and 375 nm, respectively. The methods allow rapid analysis of binary pharmaceutical formulation with accuracy, precision. The method was found to be simple, accurate, precise, economical and robust.

Vaishali DR et al[18]analyzed a novel rapid spectrophotometric method has been developed for simultaneous estimation of Serratiopeptidase and Diclofenac sodium by absorption ration method (Q-Method). The method involved solving simultaneous equations based on measurement of absorbance at two wavelengths 255 nm and 276 nm, the isosbestic point and at λmax of Diclofenac sodium respectively. Beer's law was obeyed in the concentration range 140-240 µg/ml and 14-24 µg/ml for Serratiopeptidase and Diclofenac sodium respectively.

Vishnu P et al[19] done UV spectrophotometric baseline manipulation methodology for simultaneous determination of drotaverine (DRT) and etoricoxib (ETR) in a combined tablet dosage form has been developed.Spectroscopy where the amplitudes at 274 and 351 nm were selected to determine ETR and DRT, respectively, in combined formulation and methanol was used as solvent. The results of analysis have been validated statistically .the method which were carried out by following the ICH guidelines. It has been concluded that a new simple and accurate UV

spectrophotometric baseline manipulation method was developed for simultaneous determination of drotaverine and ETR in a combined tablet dosage form has been developed.

Krishnaveni G et al[20] developed RP-HPLC validated method for rapid assay of celecoxib in tablet dosage form. Isocratic elution at a flow rate of 1.5ml/min was employed on a symmetry Chromosil C18 (250x4.6mm, 5µm in particle size) at ambient temperature. The mobile phase consisted of Methanol: ACN: 60:40 (V/V). The UV detection wavelength was 220 nm and 20µl sample was injected. The retention time for celecoxib was 3.57 min. The percentage RSD for precision and accuracy of the method was found to be less than 2%. The method was validated as per the ICH guidelines. The method was successfully applied for routine analysis of celecoxib in tablet dosage form and bulk drug.

Chaple DR et al[21] opted spectrophotometric method has been developed for simultaneous estimation of etoricoxib and paracetamol in combined dosage form using multicomponent mode of analysis. It involves the measurements of absorbance at five selected wavelengths 235 nm, 243 nm, 264 nm, 284 nm and 295 nm using methanol and hydrochloric acid (0.2 N) as a solvent. Linearity was observed in the range of 1-50 µg/mL for mixture. The recovery studies confirmed the accuracy of the proposed method. The results were validated as per ICH guidelines.

Suresh kumarS et al[22] analyzed RP-HPLC method was developed for the simultaneous estimation of Etoricoxib(ETR) and Thiocolchicoside(THC) in bulk and combined tablet dosage form. Chromatography was performed on C18 stainless steel column (InertSil ODS-3,250 mm x 4.6 mm ID, particle size 5µm), the mobile phase used was a mixture of phosphatebuffer (PH6, adjusted with orthophosphoric acid) and methanol (30:70 v/v). The wavelengthused for detection of Etoricoxib and Thiocolchicoside was 255 nm and flow rate of 1.2ml/min. The retention times were 2.506 min. and 4.600 min. for EtoricoxibandThiocolchicoside, respectively. Linearity was determined for Etoricoxib in the range of 40-80µg/ml and for Thiocolchicoside 2-6 µg/ml. The correlation coefficient (‘r’) values werefound to be >0.999. The method was validated with respect to accuracy, precision,

linearity and robustness as per the ICH Guidelines.

Krishna Gupta R et al[23]reversed-phase HPLC method has been developed and subsequently validated for simultaneous estimation of etoricoxib (ETX) and paracetamol (PCT) from their combination product. The proposed RP-HPLC method utilizes a Phenomenex® C18, 5µm, 250mm X 4.6mm i.d. column, mobile phase consisting of acetonitrile, methanol and water in the proportion of 60:15:25 (v/v/v) and UV detection at 236.0 nm using a UV detector. The described method was linear over a range of 8.3-41.5 µg mL-1 for PCT and 1-5 µg mL-1 for ETX with correlation coefficients values of 0.9999 and 0.9993, respectively.

Sanjiv Kumar et al[24]reverse phase high performance liquid chromatographic method was developed for simultaneous estimation of etoricoxib and thiocolchicoside in combined tablet dosage form. Formulation containing etoricoxib and thiocolchicoside is used as analgesic. Chromatography was performed on a 250 mm ◊ 4.6 mm, 5-µm particle size, BDS Hypersil C-18 column with trifluoroacetic acid buffer (pH 2.6) and acetonitrile (75:25, v/v) as a mobile phase. The detection of the combined dosage form was carried out at 220 nm and a flow rate employed was 1.5 mL/min. The retention times were 6.6 and 3.1 min for etoricoxib and thiocolchicoside, respectively.

SachinGholveet al[25] reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method and validate as per ICH & USP guidelines for the quantitative estimation of Etoricoxib in pharmaceutical dosage forms. The separation was conducted by using mobile phase consisting of methanol: ammonium acetate buffer:acetonitrile in the ratio (70:20:10). The separation was conducted by using Zebra Eclipse XDB-C-18 (4.6□~150□~5µm) at the flow rate of 1.0 ml/min using variable wavelength detector. The precision is exemplified by relative standard deviation of 1.15 to 1.8 %. Percentage Mean recovery was found to be in the range of 97to99%, during accuracy studies. The limit of detection (LOD) and limit of quantitiation (LOQ) was found to be 5 ng/ml and 15 ng/ml respectively.

Venkatachalam and lalitha KG[26] Developed and validated for the analysis of Thiocolchicoside and ketoprofen in tablet formulations.The method has been shown adequate separation of both ingredients from each other. The chromatographic separation was carried out on a reverse phase column-C18 (250 mm x 4.6 mm,5µ), with a mobile phase consisting of 0.05 M ammonium acetate buffer (adjusted to pH 6 with glacial acetic acid), acetonitrile and methanol in the ratio (50:30:20,v/v) at a flow rate of 1.2ml/min and UV detection at 310 nm. The average recovery of the method is 98.88% and 100.07% for thiocolchicoside and ketoprofen respectively. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operator indicating that the method is sufficiently robust and rugged.

Vaishalia et al[27] Reversed phase liquid chromatography method (HPLC) method was developed for the quantitative estimation of celecoxib, a selective COX-2 inhibitor in capsule formulations. The drug was chromatographed on a reversed-phase C-18 column. Eluents were monitored at a wavelength of 220 nm using a mixture (600:400:1:1) of acetonitrile, Water, Triethylamine and Orthophosphoric acid. The retention time of Celecoxib was found to be 9.5 minutes. The flow rate of the mobile phase was 1.0 ml/min at room temperature. The percentage recovery lies in the range of 99.53%–99.75%. Solution concentrations were measured on a weight basis to avoid the use of an internal standard.

Prinesh N. Patelet al[28]RP-HPLCmethod for simultaneous determination of 9 NSAIDs (paracetamol, salicylic acid, ibuprofen, naproxen, aceclofenac, diclofenac, ketorolac, etoricoxib, and aspirin) and their commonly prescribed combination drugs (thiocolchicoside, moxifloxacin, clopidogrel, chlorpheniramine maleate, dextromethorphan, and domperidone) was established. The separation was performed on Kromasil C18 (250 × 4.6 mm, 5 μm) at 35 Degree celcius using 15mM phosphate buffer pH 3.25 and acetonitrile with gradient elution at a flow rate of 1.1 mL/min. Calibration curves were linear with correlation coefficients of determination ($r2$) >0.999. Limit of detection (LOD) and Limit of quantification (LOQ) ranged from 0.04 to 0.97 μg/mL and from 0.64 to 3.24 μg/mL, respectively.

Gajera JB et al[29]spectroscopic method was developed for simultaneous estimation of Tizanidine Hydrochloride and Rofecoxib using Ratio Derivative Method. Ratio was taken using divisor. Divisor for 0.5-2.5µg/ml Tizanidine Hydrochloride was 6µg/ml of Rofecoxib and for 6-30µg/ml Rofecoxib was 4µg/ml of Tizanidine Hydrochloride. After divisor spectra were converted to First derivative. Tizanidine Hydrochloride showed zero crossing point at 253.00nm while Rofecoxib showed zero crossing point at 316.00nm. The absorbance was measured at 316.00nm for Tizanidine Hydrochloride and 253.00nm for Rofecoxib and calibration curves were plotted as absorbance versus concentration, respectively. The limit of quantification was 0.052µg/ml and 0.39µg/ml for Tizanidine Hydrochloride and Rofecoxib, respectively.

Sekar Vet al[30] reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Diacerein and Aceclofenac in combination. The separation was carried out using a mobile phase consisting of dipotassium hydrogen phosphate buffer of pH 4.5, Acetonitrile and methanol in the ratio of 40: 40: 20. The column used was Phenomenex Luna C18, 5µ, 250 mm × 4.6 mm id with flow rate of 1.0 ml / min using UV detection at 265 nm. The described method was linear over a concentration range of 80-120 µg/ml and 160-240 µg/ml for the assay of Diacerein and Aceclofenac respectively.

Raju Chandra et al[31] developed and validated the stable isocratic reverse phase high performance liquid chromatography and UV-Visible method for regulate the quality and estimation of Etoricoxib drug content from the marketed pharmaceutical tablets. The reverse phase high performance liquid chromatography and UV-Visible method validation for Etoricoxib have done followed by assay methodology. The retention time (Rt) of Etoricoxib was 10.0 min with the flow rate of 1.0 mL/min at wave length 272 nm. The linearity of method was validated for Etoricoxib drug content in the range of 5-100 µg/mL with correlation coefficient (r) values 0.997 and 0.998 for RP- HPLC and UV-Visible, respectively. This method is stable and validated to assay analysis. Thus, the validated method is can be successfully applied to routine analysis for regulate the quality.

Syal PK et al[32]probed RP-HPLC-PDA method has been developed for the simultaneous estimation of DrotaverineHCl (DRT) and Etoricoxib (ETR) in tablet formulations. The chromatographic separation was achieved on Waters Kromosil C18 column (250 mm x 4.6 mm, 5.0 µ particle size) using methanol: THF: acetatebuffer (51:09:40 v/v) pH adjusted to 6.0 with acetic acid, flow rate was 0.9ml/min and column was maintained at 55 0C. Quantification and linearity was achieved at 244 nm over the concentration range of 1.6 – 80µg/ml for DrotaverineHCl and 1.8 – 90µg/ml for Etoricoxib. The method was validated for specificity, linearity, accuracy, precision, LOD, LOQ and robustness. The proposed method was optimized and validated as per the ICH guidelines.

Manish kumaret al[33]reverse phase high performance liquid chromatographic method was developed and validated for the determination of etoricoxib in bulk and tablet dosage forms. It was found that the excipient in the tablet dosage forms does not interfere in the quantification of active drug by proposed method. The HPLC separation was carried out by reverse phase chromatography on Shimadzu HPLC, 10-At detector with hypersil ODS C18 Column 250 X 4.6 mm (particle size of 5µ) and constant flow pump. Rheodyne injector with 20 µlloop with a mobile phase composed in the ratio acetonitrile: (0.05M) KH2PO4 buffer (50:50) at flow rate 1.8 ml /min. The detection was monitored at 283nm.

Ashok R Parmar et al[34]developed and validated for Simultaneous estimation of Aceclofenac and Serratiopeptidase in tablet dosage form using double beam UV Spectrophotometer of thermo Electron Corporation (Heλlos α) with (Ethanol + Water) as a solvent. Absorption maxima of Aceclofenac and Serratiopeptidase in Ethanol diluted with water was found to be 316 nm and 375 nm, respectively. Beer's law was obeyed in the concentration range 30-70 µg/ml for Aceclofenac and 100-300 µg/ml Serratiopeptidase. The mean recoveries obtained for Aceclofenac and Serratiopeptidase were 99.193 % and 99.153 % respectively. The LOD and LOQ for Aceclofenac were found to be 2.334µg/ml and 7.074µg/ml and for Serratiopeptidase 12.50µg/ml and 37.88µg/ml respectively.

Aayushiet al[35]RP-HPLC method was developed for the estimation of Etoricoxib in pharmaceutical dosage forms. Method development incorporated the optimization of stationary phase (column), mobile phase composition and other chromatographic conditions. The method was carried out on Symmetry (Waters) RP-C18 (250 x 4.6 mm), 5 µm column using a mixture of Phosphate buffer: Acetonitrile in the ratio/ composition 30:70 v/v. The mobile phase was pumped at a flow rate of 1ml/min and the detection was carried out at 220 nm. The retention time of the drug was 9.585 min. Method validation produced excellent results for specificity, linearity, precision, intermediate precision, accuracy, limit of detection and limit of quantitation and robustness. The linearity was found within concentration range of 70 ppm to 130 ppm with correlation coefficient of 0.998145.

VijayaKuchana et al[36]Reverse Phase -HPLC method was developed for the estimation of Etoricoxib in its bulk and tablet dosage forms, using symmetry C18 column (4.6×150mm, 5.0µm particle size) and a mobile phase of Methanol:Water in the ratio of 70:30(v/v),at a flow rate of 1.2ml/min with UV detection at 235nm.The Retention time (TR) of Etoricoxib is 2.293 min. The proposed method is validated for system suitability, specificity, linearity, accuracy, precision, sensitivity, ruggedness and robustness as per ICH guidelines.

KalpanaNekkala et al[37]developed isocratic reverse phase liquid chromatography (RP-LC) method has been developed and subsequently validated for the determination of Celecoxib in Bulk and its pharmaceutical formulation. Separation was achieved with a Symmetry RP-18 ((Make: Waters Corporation; 75 mmx4.6 mm I.D; particle size 5 µm)) Column and Potassium dihydrogen phosphate monohydrate buffer (pH adjusted to 3.0 with diluted orthophosphoric acid): methanol: acetonitrile (400:400:200) v/v as eluent at a flow rate of 0.8 mL/min. UV detection was performed at 225nm. The method is simple, rapid, and selective. The described method of Celecoxib is linear over a range of 25 µg/mL to 75 µg/mL. The method precision for the determination of assay was below 1.0% RSD.

Pattan SRet al[38] *developed*reverse phase high-pressure liquid chromatographic method has been developed for the simultaneous estimation of Paracetamol and Etoricoxib from pharmaceutical formulation. The method was carried out on an inertsil ODS, 5µ, C8-3 column, with a mobile phase consisting of methanol: acetonitrile: phosphate buffer pH 3.5 (40:20:40 v/v) at a flow rate of 1.0 ml/min. Detection was carried out at 242. The retention time of Paracetamol and Etoricoxib were 3.27, 6.12 min. respectively.

Santosh R et al[39] quantitative estimation of Celecoxib using analytical grade methanol as solvent and acetyl chloride as the reagent for acetylation of Celecoxib has been developed. Acetyl derivative of Celecoxib obeys Beer's law in concentration range 20-40 µg/ml at 270nm as absorption maximum. The recovery studies ascertained accuracy of purposed method and result validated according to ICH guidelines. The result of analysis has been validated statistically by recovery studies. This method was successfully carried out for the estimation of Celecoxib in capsule dosage form without the interference of common excipients.

Revathiet al[40] AssignedA new, simple and sensitive spectrphotometric method in Ultraviolet region has been developed for the determination of Celecoxib in bulk and in pharmaceutical formulations. Celecoxib, exhibits absorption maxima at 255 nm with apparent molar absorptivity 1.7848 X 104 L/mol.cm in chloroform. Beer's law was found to be obeyed in the concentration range of 2-10ug/mL. The method is accurate, precise and economical. This method is extended to pharmaceutical preparations.

AIM AND OBJECTIVE

3. AIM AND OBJECTIVE

- The aim and objective of the present study is a simultaneous estimation of Etoricoxib and Serratiopeptidase present in Multi component tablet dosage form by RP-HPLC is considered to be most suitable.
- Combination of Etoricoxib and Serratiopeptidase is one of new combination approved by CDSCO (2010). Etoricoxib is officially reported in JP and Serratiopeptidase is scientific validation not reported in any Pharmacopoeia. Etoricoxib and Serratiopeptidase combinations are scientifically not reported on official records.
- In our deep literature survey reveals that, not a single analytical method is reported for the determination of these drugs in combination and also during the research not following standard guidelines.
- Based on the above reasons I have selected topic on “Simultaneous Estimation of Etoricoxib and Serratiopeptidase in RP-HPLC method”.
- Also required above scientific validation for Etoricoxib and Serratiopeptidase combination as per ICH guidelines with the help of RP-HPLC, Which will be useful for develop simple, sensitive, accurate, precise and economical spectroscopic as well as chromatographic analytical methods for the estimation of these two drugs in combination.

PLAN OF WORK

Plan of the work

- To perform the literature review
- To develop simultaneous equation method for the estimation of Etoricoxib and Serratiopeptidase in tablet dosage
- To develop and validate first order derivative method for estimation of Etoricoxib and serratiopeptidase
- To develop and validate area under curve method for estimation of Etoricoxib and serratiopeptidase
- To develop and validate RP-HPLC method for estimation of Etoricoxib and serratiopeptidase

METHODOLOGY

4. DEVELOPMENT OF SIMULTANEOUS EQUATIONS METHOD FOR ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE

4.1 EXPERIMENTAL

4.1.1 Apparatus

- A double beam UV-visible Spectrophotometer (Shimadzu, UV-1700, Japan), attached to a computer software UV probe 2.0, with a spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells.
- Analytical balance (CP224S, Sartorius, Germany)
- Ultrasonic cleaner (Frontline FS 4, Mumbai, India)
- Corning volumetric flasks and pipettes of borosilicate glass

4.1.2 Reagents and Materials

- Etoricoxib (ETO) and Serratiopeptidase (SER) were kindly supplied as a gift samples from Sun Pharma Ltd, Vadodara, Gujarat, India and Acme Pharmaceuticals Ltd, Ahmedabad, Gujarat, India, respectively.
- The tablet dosage containing 50 mg ETO and 4 mg SER was prepared in the laboratory.
- AR grade methanol (S.D. Fine Chemical Ltd., Mumbai, India.)
- Whatman filter paper no. 41 (Whatman International Ltd., England)

4.1.3 Preparation of Solutions

4.1.3.1 Preparation of Standard Stock Solutions

Accurately weighed portion of ETO (10 mg) and SER (10 mg) were transferred in a separate 100 ml of volumetric flasks. Methanol (50 ml) was added to 100 ml volumetric flask containing (10 mg) SER and sonicated for 45 min. The solution of SER was filtered through whatman filter paper no. 41 and volume of the both the volumetric flasks containing SER and ETO were adjusted up to the mark with methanol.

4.1.3.2 Preparation of Tablet dosage

500 mg of tablet dosage was prepared by using ETO (90mg) and SER (10mg) and excipients like Microcrystaline cellulose, Starch, Magnesium stearate and Talc.

4.1.3.3 Preparation of Sample Solution

The tablet dosage containing ETO (50 mg) & SER (4 mg) was then transferred to 100 ml volumetric flask containing 50 ml methanol and sonicated for 20 min. The solution was filtered through Whatman filter paper No. 41 and the volume was adjusted up to the mark with methanol. From this solution 0.5 ml was taken in to a 10 ml volumetric flask and the volume was adjusted up to mark with methanol to get a final concentration of ETO (25 µg/ml) and SER (2 µg/ml).

4.1.4 Method Development

4.1.4.1 Determination of Wavelength having Maximum Absorbance

Standard solutions of ETO (10 µg/ml) and SER (10 µg/ml) were scanned in the range of 200 to 400 nm for the determination of wavelength having maximum absorbance. The wavelength having maximum absorbance for ETO and SER were selected.

4.1.4.2 Preparation of Calibration Curves

From standard stock solutions, aliquots of ETO (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0 ml) and SER (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0 ml) were transferred in a series of 10 ml volumetric flasks. The volume was adjusted to the mark with methanol and mixed.

The absorbances of all the solutions were measured at 255 nm and 288 nm against methanol as blank. The calibration curves were prepared by plotting graph of absorbance Vs concentration for both ETO and SER.

4.2 METHOD VALIDATION

4.2.1 Linearity

Linearity was observed in a concentration range of 2-30 µg/ml for both ETO and SER. The calibration curves were constructed by plotting the graph of absorbance Vs concentration.Range is the interval between upper and lower concentration of analyte for which it has been demonstrated that the analytical method has suitable level of precision, accuracy and linearity. The range for the method was observed in a concentration range of 2-30 µg/ml for both ETO and SER. For the evaluation of the range accurately measured standard working solutions of ETO (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0 ml) and SER (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0 ml) were pipette out into a separate series of 10 ml volumetric flasks. The volume was adjusted with methanol and absorbances of all the solutions were measured at 255.0 nm and 288.0 nm against methanol as blank.

4.2.2 Method Precision (Repeatability)

The precision of the instrument was checked by repeated scanning and measuring the absorbance of solutions (n = 6) of ETO and SER (10 µg/ml for both drugs) without changing the parameters of the Simultaneous Equations Method. The results are reported in terms of relative standard deviation (% RSD).

4.2.3 Intermediate Precision (Reproducibility)

The intraday and interday precision of the proposed method was evaluated by analyzing the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of standard solutions of ETO and SER (10, 14 and 18 µg /ml). The results were reported in terms of relative standard deviation (% RSD).

4.2.4 Limit of Detection (LOD) & Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) of the method were calculated by using the following equations.

LOD = 3.3 X σ/S

LOQ = 10 X σ/S

Where, σ = the standard deviation of the response

S = slope of the calibration curve

4.2.5 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recoveries of ETO and SER by the standard addition method in two steps. Known amounts of standard solutions of ETO (7.5, 10, 12.5 µg/ml) and SER (6, 8, 10 µg/ml) were added at 75%, 100% and 125% levels to prequantified sample solutions of ETO (10 µg/ml) and SER (8 µg/ml).

4.3 ANALYSIS OF DRUGS IN TABLET DOSAGE

Tablet dosage of ETO and SER was prepared in laboratory**.** The absorbance of sample solution was measured against methanol as blank at 255 and 288 nm for quantification of ETO and SER, respectively. The amount of ETO and SER present in the sample solutions were determined by solving the simultaneous equations.

Concentration of both drugs is calculated by solving the simultaneous equations 1 & 2.

Cx = (A2 aY1 – A1 aY2) / (aY1 aX2 - aY2 aX1) ----- (1)

Cy = (A1 aX2 - A2 aX1) / (aY1 aX2 - aY2 aX1) ------ (2)

Where, A1, A2= Absorbances of mixture at λ1 & λ2 respectively,

ax1 =Absorpitivity of first drug at λ1,

ax2 = Absorpitivity of first drug at λ2,

ay1=Absorpitivity of second drug at λ1, ay2 = Absorpitivity of second drug at λ2.

4.4 RESULTS AND DISCUSSION

4.4.1 Method Development

The working standard solutions of ETO and SER were prepared separately in methanol. They were scanned in the wavelength range of 200-400 nm. Maximum absorbance was obtained at 255 nm and 288 nm for ETO and SER, respectively.

These two wavelengths were employed for the determination of ETO and SER. Overlain spectra of both the drugs are shown in Figure 4.1.

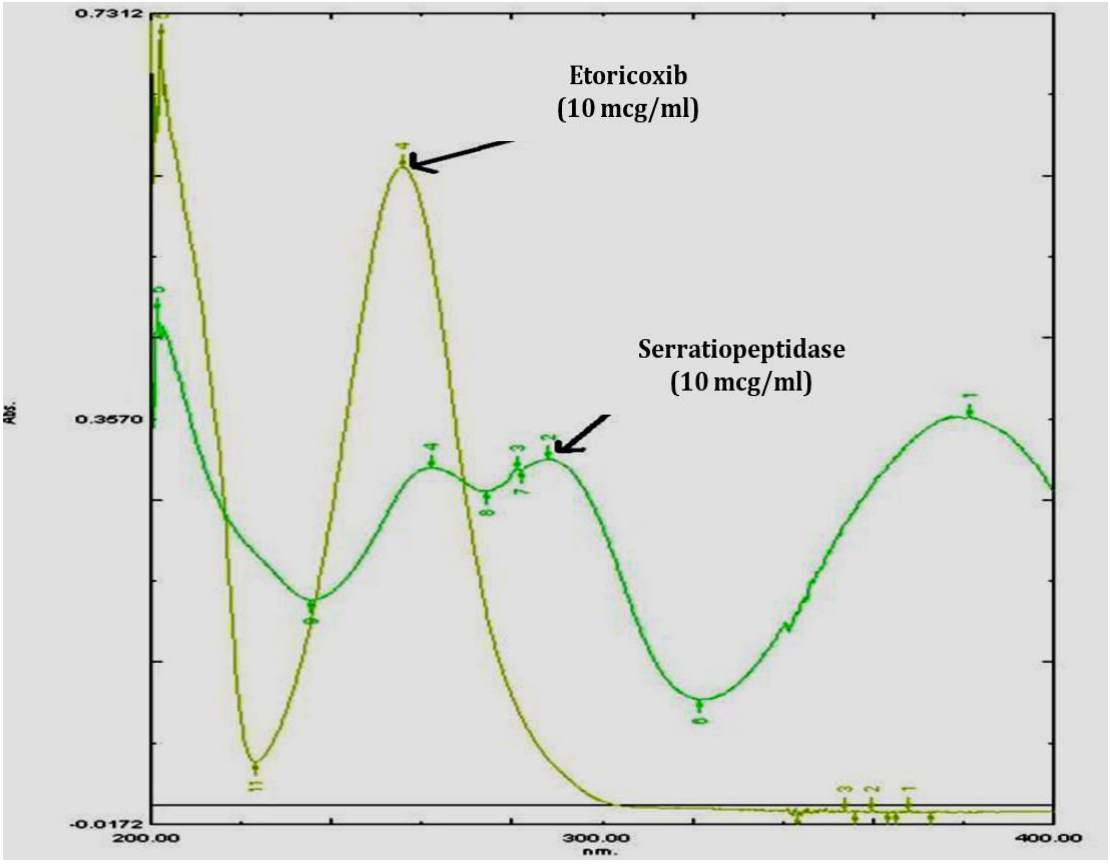


Figure 4.1: Overlain UV Absorption Spectra of ETO (10 µg/ml) and SER (10 µg/ml) in methanol

4.4.2Validation of the proposed method

4.4.2.1 Linearity

Calibration range was observed in the concentration range of 2-30 µg /ml for both ETO and SER. The calibration curves at different wavelengths are shown in Figure. 4.2, 4.3, 4.4, 4.5

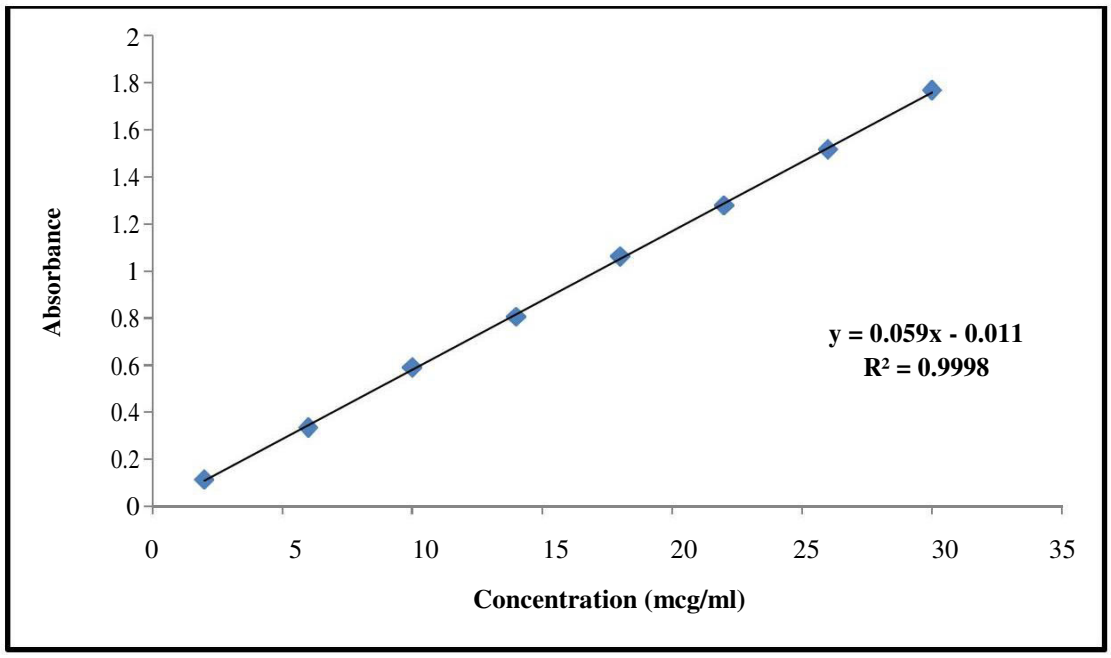


Figure 4.2: Calibration Curve of ETO at 255 nm

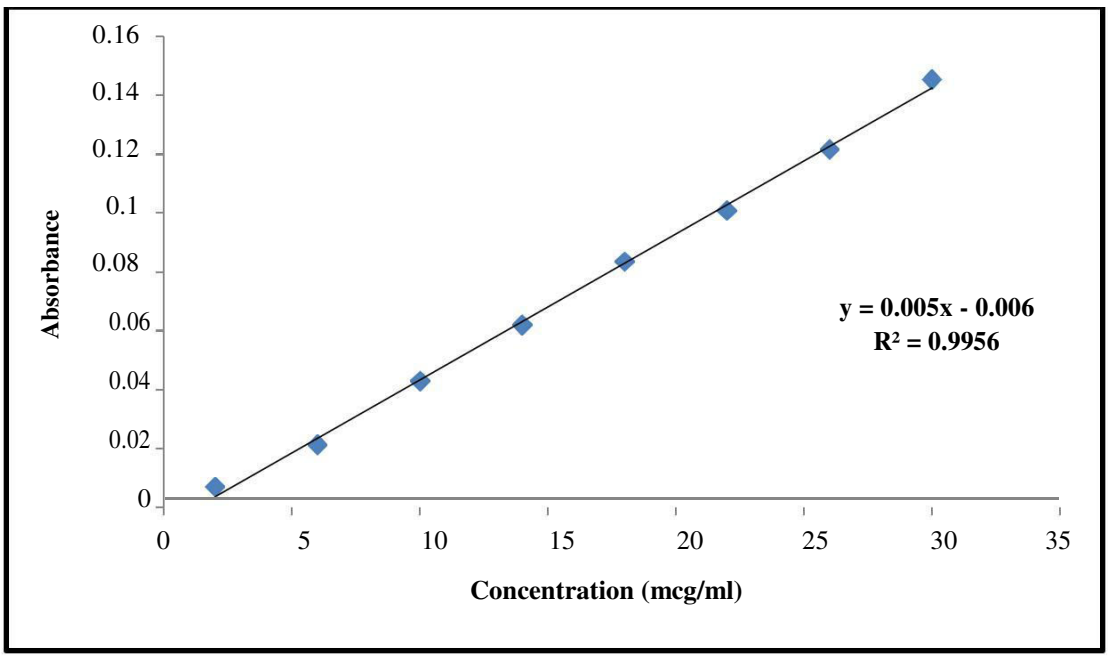


Figure 4.3: Calibration Curve of ETO at 288 nm

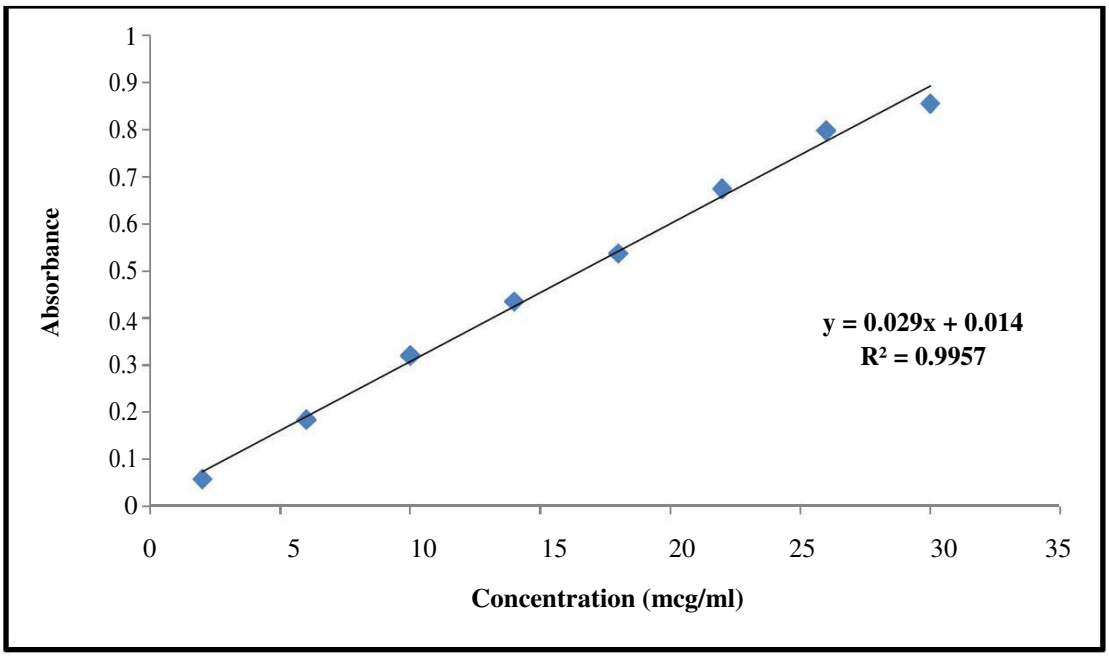


Figure 4.4: Calibration Curve of SER at 288 nm

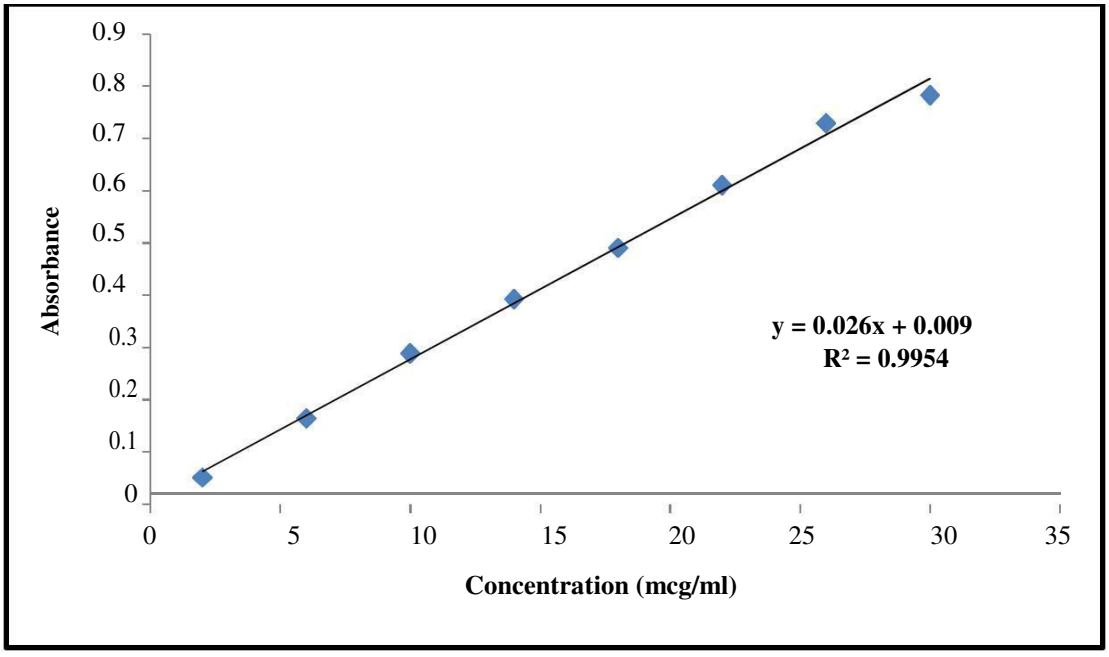


Figure 4.5: Calibration Curve of SER at 255 nm

Table 4.1: Regression analysis data and summary of validation parameters for the proposed method

PARAMETERS		ETO		SER	
Wavelength (nm)		255	288	288	255
Beer's law limit (µg /ml)		2-30	2-30	2-30	2-30
Regression equation (y = mx + c) Slope (m) Intercept (c)		y = 0.059x - 0.011 0.059 -0.011	y = 0.005x – 0.006 0.005 -0.006	y = 0.029x + 0.014 0.029 0.014	y = 0.026x - 0.009 0.026 -0.009
Correlation coefficient (r^2)		0.9998	0.9956	0.9957	0.9954
LOD(a) (µg/ml)		0.36	0.43	0.26	0.24
LOQ(b) (µg /ml)		1.10	1.29	0.79	0.72
Sandell's sensitivity (µg/cm^2/0.001 absorbance unit)		0.01725	0.1449	0.03299	0.03640
Molar extinction co-efficient (l mol^{-1} cm^{-1})		17145.69	20413.45	11270.60	10213.52
Repeatability (% RSDc , n = 6)		0.34	0.54	0.17	0.20
Precision (% RSD, n = 3)	Intraday	0.21-0.49	0.42-0.95	0.13-0.32	0.23-0.47
	Interday	0.16-0.34	0.40-0.72	0.12-0.24	0.11-0.33
Accuracy ± S. D (% Recovery, n = 3)		99.5 ± 0.26		99.87 ± 0.35	

[a]LOD = Limit of detection. [b]LOQ = Limit of quantification. [c]RSD = Relative standard deviation. [d]S. D. is Standard deviation

4.4.2.2 Method Precision (Repeatability)

The RSD values of ETO were found to be 0.34 and 0.54 % at 255 and 288 nm respectively. The RSD values of SER were found to be 0.20 and 0.17 % at 255 and 288 nm (Table 4.2). Low values of RSD indicate that proposed method is repeatable.

Table 4.2: Repeatability Data of ETO and SER by the proposed method

Concentration (ETO: SER) (10:10 µg /ml)	ETORICOXIB		SERRATIOPEPTIDASE	
	255 nm	**288 nm**	**255 nm**	**288nm**
1	0.5884	0.0428	0.2889	0.3195
2	0.5892	0.0431	0.2877	0.3201
3	0.5916	0.0432	0.2892	0.3197
4	0.5922	0.0435	0.2881	0.3204
5	0.5930	0.0433	0.2887	0.3210
6	0.5931	0.0431	0.2881	0.3199
Mean	0.5912	0.0431	0.2884	0.3201
S.D.	0.0020	0.0002	0.0006	0.0005
% RSD	0.34	0.54	0.20	0.17

4.4.2.3 Intermediate Precision (Reproducibility)

The RSD values of ETO for interday (0.21-0.49 % and 0.42-0.95 %) and intraday (0.16-0.34 % and 0.40-0.72 %) at 255 and 288 nm, respectively and the RSD values of SER for interday (0.13-0.32 % and 0.23-0.47 %) and intraday (0.12-0.24 % and 0.11-0.33 %) at 288 nm and 255 nm, reveal that the method is precise.

4.4.2.4 LOD and LOQ

LOD and LOQ values for ETO were found to be 0.36 and 1.10 µg/ml, 0.43 and 1.29 µg/ml at 255 and 288 nm, respectively. Where, LOD and LOQ values for SER were found to be 0.26 and 0.79 µg/ml, 0.24 and 0.72 µg/ml at 288 nm and 255 nm, respectively. Low values of LOD & LOQ indicate that the method is sensitive. (Table 4.1)

4.4.2.5 Accuracy (% Recovery)

The recovery experiments were performed by the standard addition method. The mean recoveries were found to be 99.50 ± 0.26 and 99.87 ± 0.35 for ETO and SER respectively. The recoveries results indicate that the proposed method is accurate. Results of recovery studies are shown in Table 4.3

Table 4.3: Recovery Data for the proposed method (n=3)

Drug	Amount present in mixture (µg/ml)	Amount present in mixture (µg/ml)	% Recovery ± S. D. (n = 3)
ETO	10	75	98.11 ± 0.11
	10	100	101.5 ± 0.36
	10	125	98.95 ± 0.31
SER	8	75	99.15 ± 0.60
	8	100	98.40 ± 0.39
	8	125	102.0 ± 0.06

4.4.3 Assay

The proposed validated method was successfully applied to determine ETO and SER in tablet dosage. Results are given in Table 4.4. No interference of the excipients with the absorbance of analyte of interest appeared; hence the proposed method is suitable for the routine analysis of ETO and SER in tablet dosage.

Table 4.4: Analysis of ETO and SER in tablet dosage by the proposed method (n=6)

Synthetic mixture	Label claim (mg)		Amount found (mg)		% Label claim (mg) (n = 6)	
	ETO	SER	ETO	SER	ETO	SER
1	50	4	50.94	4.10	101.9	102.5
2	50	4	51.02	4.06	102.0	101.5
3	50	4	51.00	4.10	102.0	102.5
4	50	4	51.08	4.04	102.2	101.0
5	50	4	48.74	3.94	97.48	98.50
6	50	4	51.04	4.08	102.1	102.1
MEAN			50.64	4.05	101.3	101.4
S.D.			0.93	0.06	1.86	1.52

4.5 CONCLUSION

Based on the results obtained from the analysis using proposed method, it can be concluded that the method has linear response in the range of 2-30 µg/ml for both ETO and SER. The results of the analysis of tablet dosage by the proposed method are highly reproducible and reliable and are in good agreement with label claim of the drugs. The additive present in the tablet dosage did not interfere in the analysis. So the method can be used for the routine analysis of drugs in combination.

5.DEVELOPMENT AND VALIDATION OF AREA UNDER CURVE METHOD FOR ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE

5.1EXPERIMENTAL

5.1.1 Apparatus same as 4.1.1

5.1.2 Reagent and Materials same as 4.1.2

5.1.3 Preparation of solutions

5.1.3.1 Preparation of Standard Stock Solution same as 4.1.3.1

5.1.3.2 Preparation of Tablet dosage same as 4.1.3.2

5.1.3.3 Preparation of Sample Solution same as 4.1.3.3

5.1.4 Method Development

5.1.4.1 Determination of analytical wavelength

Standard solutions of ETO (10 µg/ml) and SER (10 µg/ml) were scanned in the range of 200 to 400 nm for the determination of wavelength range having maximum area under curve. ETO shows 250-260 nm and SER shows 285-295 nm as the wavelength range having maximum area under curve.

5.1.4.2 Preparation of Calibration Curves

From the standard stock solutions, aliquots of ETO (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 ml) and SER (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 ml) were transferred in a series of 10 ml volumetric flasks. The volume was adjusted to the mark with methanol and mixed.

The areas under curve of all the solutions were measured at 250-260 nm and 285-295 nm against methanol as blank. The calibration curves were prepred by plotting the graph of area under curve Vs concentration.

5.2METHOD VALIDATION

5.2.1Linearity

Linearity was observed in a concentration range of 2-26 µg/ml for both ETO and SER. The calibration curves were constructed by plotting the graph of area under curve Vs concentration.Range is the interval between upper and lower concentration of analytic for which it has been demonstrated that the analytical method has suitable level of precision, accuracy and linearity. The range for the method was observed in a concentration range of 2-26 µg/ml for both ETO and SER. For the evaluation of the range accurately measured standard working solutions of ETO (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 ml) and SER (0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6 ml) were pipette out into separate series of 10 ml volumetric flasks. The volume was adjusted with methanol and area under curve of all the solutions were measured at 250-260 nm and 285-295 nm against methanol as blank.

5.2.2 Method Precision (Repeatability)

The precision of the instrument was checked by repeated scanning and measuring the absorbance of solutions (n = 6) of ETO and SER (10 µg/ml for both drugs) without changing the parameters. The results are reported in terms of relative standard deviation (% RSD).

5.2.3 Intermediate Precision (Reproducibility)

The intraday and interday precision of the area under curve method was evaluated by analyzing the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of standard solutions of ETO and SER (10, 14, and 18 µg /ml). The results were reported in terms of relative standard deviation (% RSD).

5.2.4 Limit of Detection (LOD) & Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) of the method were calculated by using the following equations.

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where, σ = the standard deviation of the response

S = slope of the calibration curve

5.2.5 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recoveries of ETO and SER by the standard addition method in two steps. Known amounts of standard solutions of ETO (7.5, 10, 12.5 µg/ml) and SER (6, 8, 10 µg/ml) were added at 75%, 100% and 125% levels to pre-quantified sample solutions of ETO (10 µg/ml) and SER (8 µg/ml).

5.3 ANALYSIS OF DRUGS IN TABLET DOSAGE

Tablet dosage of ETO and SER was prepared in laboratory**.** The area under curve of sample solution was measured against methanol as blank at 250-260 and 285-295 nm for quantification of ETO and SER, respectively. The amount of ETO and SER present in the sample solutions were determined by solving following AUC equations.

$$Cx = (A2\ aY1 - A1\ aY2) / (aY1\ aX2 - aY2\ aX1)$$

$$Cy = (A1\ aX2 - A2\ aX1) / (aY1\ aX2 - aY2\ aX1)$$

Where, Cx = Concentration of EPE,

Cy = Concentration of LOR,

A1 = Area at 250-260 nm,

A2 = Area at 285-295 nm,

aX1 and aY1 are AUC constants of ETO and SER respectively at 250-260 nm, $aX_{nm}2$ and aY2 are AUC constants of ETO and SER respectively at 285-295

AUC constant = Area/ concentration in gm/l

5.4 RESULTS AND DISCUSSION

5.4.1Method Development

The working standard solutions of ETO and SER were prepared separately in methanol. Maximum area under curve was obtained at 250-260 nm and 285-295 nm for ETO and SER, respectively. These two wavelength ranges were employed for the determination of ETO and SER. Overlain spectra of both the drugs are shown in Figure 5.1.

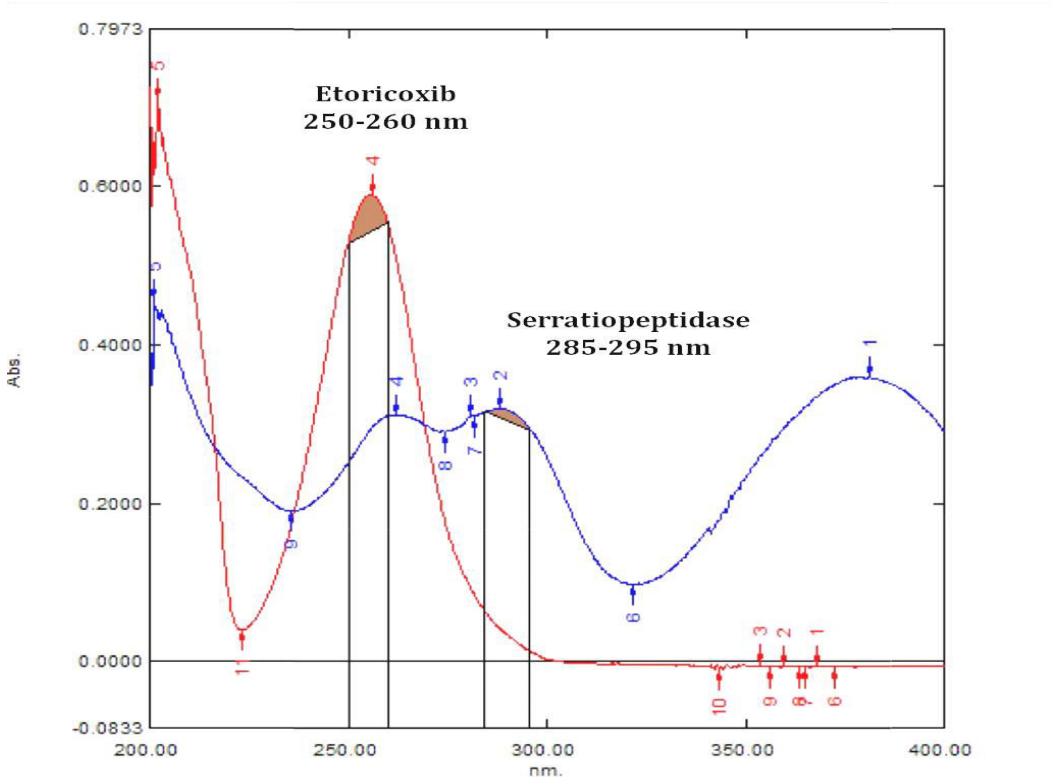


Figure 5.1: Overlain AUC spectra of ETO (10 µg/ml) and SER (10 µg/ml) in methanol

5.4.2 Validation of the proposed method

5.4.2.1 Linearity

Calibration range was observed in the concentration range of 2-26 µg /ml for both ETO and SER. The calibration curves at different wavelength ranges are shown in Figure 5.2, 5.3, 5.4, 5.5.

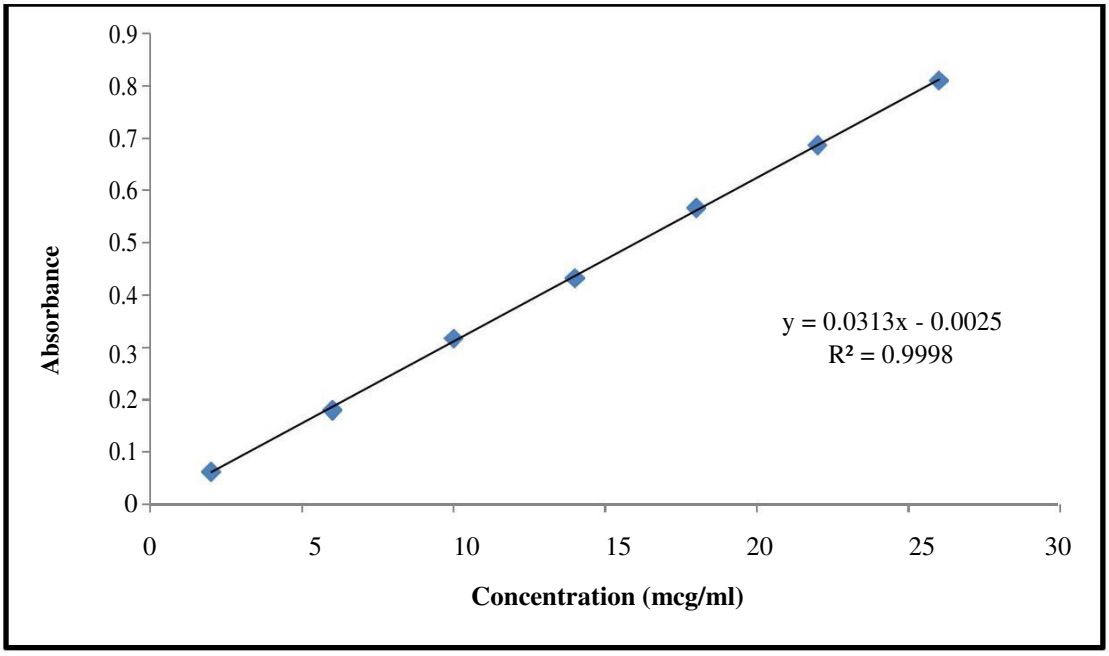


Figure 5.2: Calibration Curve of ETO at 250-260 nm

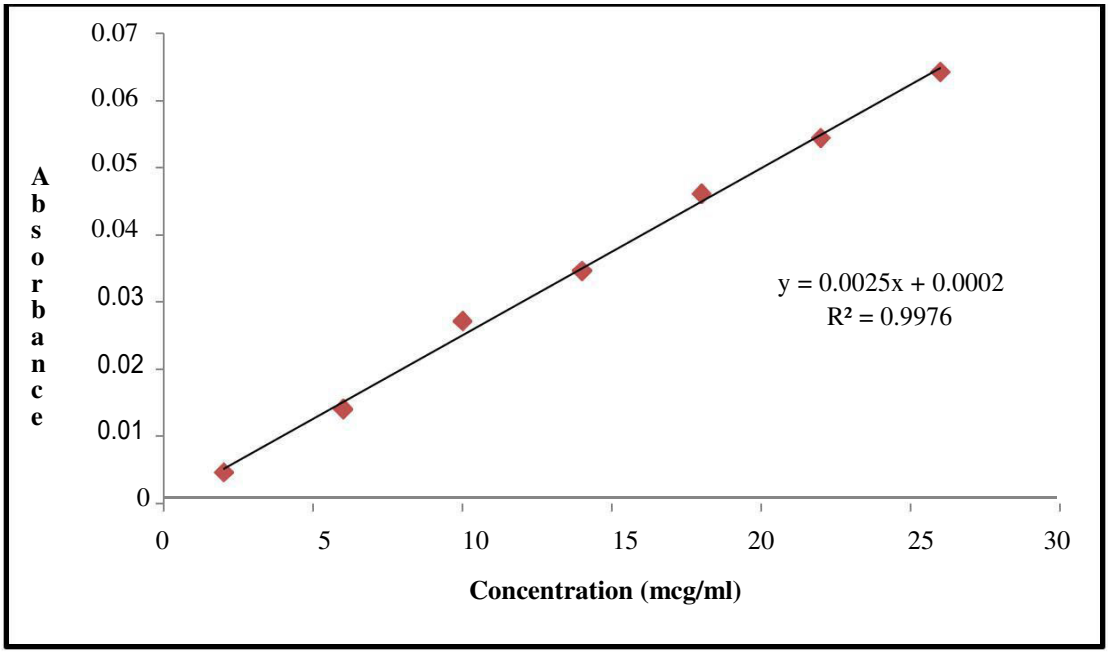


Figure 5.3: Calibration Curve of ETO at 285-295 nm

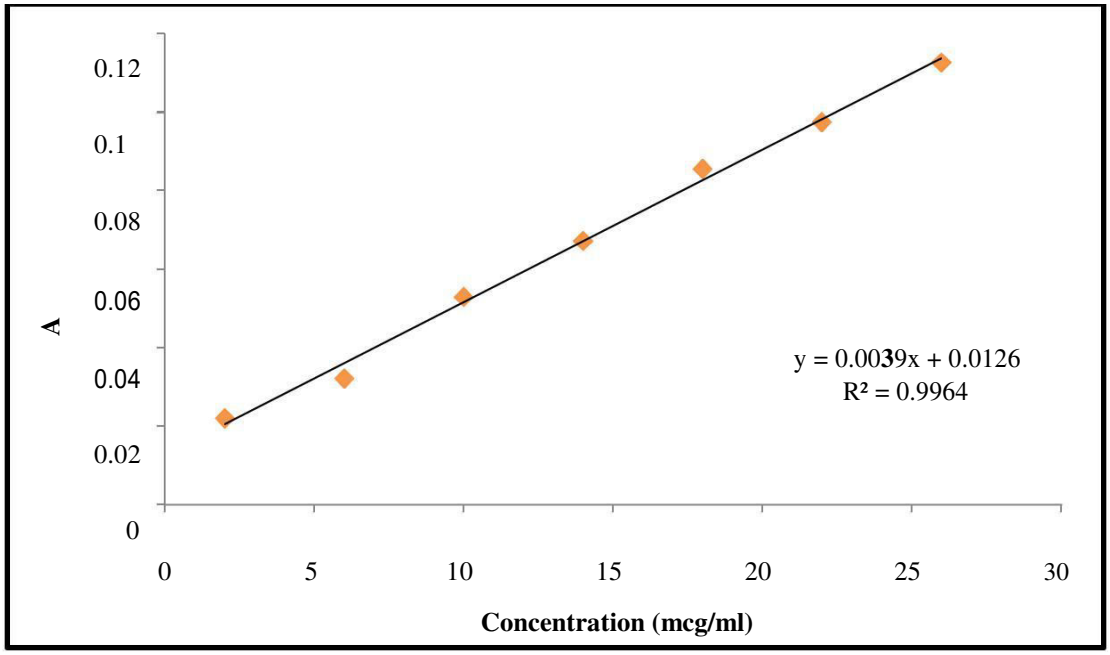


Figure 5.4: Calibration Curve of SER at 250-260 nm

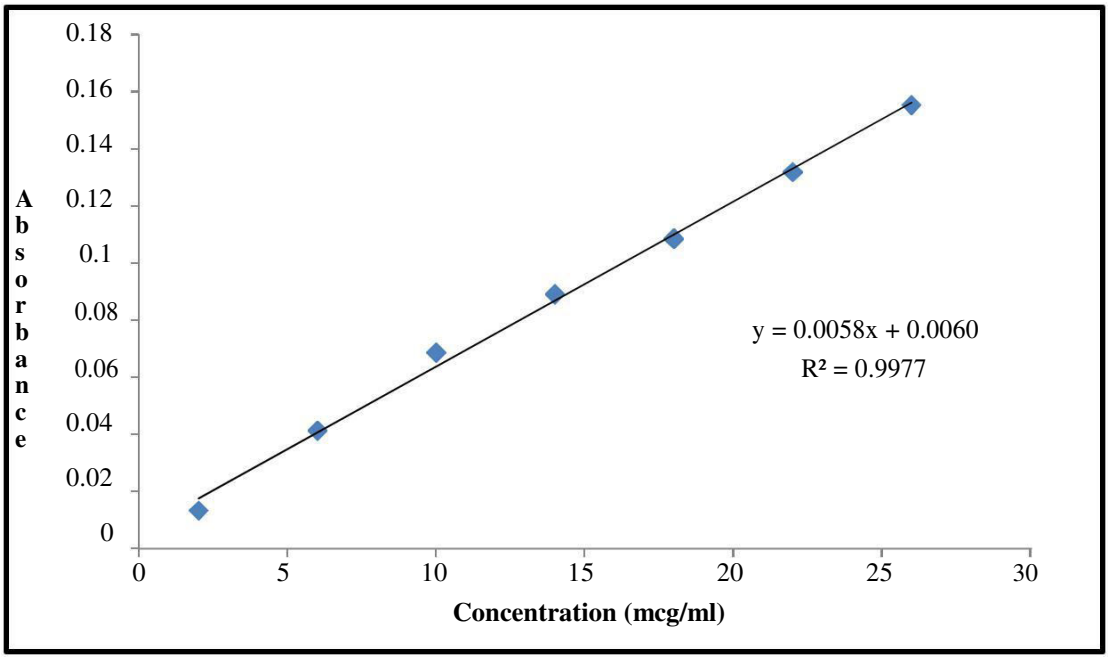


Figure 5.5: Calibration Curve of SER at 285-295 nm

Table 5.1: Regression analysis data and summary of validation parameters for the proposed method

PARAMETERS		ETO		SER	
Wavelength (nm)		250-260	285-295	285-295	250-260
Beer's law limit (µg /ml)		2-26	2-26	2-26	2-26
Regression equation (y = mx + c) Slope (m) Intercept (c)		y = 0.0313x - 0.0025 0.0313 -0.0025	y = 0.0025x + 0.0002 0.0025 0.0002	y = 0.0058x + 0.0060 0.0058 0.0060	y = 0.0039x + 0.0126 0.0039 0.0126
Correlation coefficient (r^2)		0.9998	0.9976	0.9977	0.9964
LOD (µg/ml)		0.39	0.55	0.59	0.59
LOQ (µg /ml)		1.18	1.66	1.80	1.78
Repeatability (% RSD, n = 6)		0.51	1.80	1.26	1.85
Precision (% RSD, n = 3)	Interday	0.54-1.09	1.20-1.92	0.97-1.85	1.24-1.90
	Intraday	0.19-0.71	0.37-1.32	0.55-1.36	0.87-1.37
Accuracy ± S. D. (% Recovery, n = 3)		99.82 ± 0.41		100.2 ± 0.93	

LOD = Limit of detection, LOQ = Limit of quantification,

RSD = Relative standard deviation, S. D. = Standard deviation

5.4.2.2 Method Precision (Repeatability)

The RSD values of ETO were found to be 0.51 and 1.80 % at 250-260 and 285-295 nm respectively. The RSD values of SER were found to be 1.85 and 1.26 % at 250-260 and 285-295 nm (Table 5.2). Low values of RSD indicate that proposed method is repeatable.

Table 5.2: Repeatability data for ETO and SER

Concentration (ETO: SER) (10:10 µg /ml)	ETO		SER	
	250-260 nm	285-295 nm	250-260 nm	285-295nm
1	0.3139	0.0271	0.0521	0.0681
2	0.3182	0.0274	0.0537	0.0677
3	0.3172	0.0279	0.0526	0.0700
4	0.3145	0.0270	0.0530	0.0692
5	0.3165	0.0265	0.0515	0.0685
6	0.3161	0.0276	0.0541	0.0694
Mean	0.3160	0.0272	0.0528	0.0688
S.D.	0.0016	0.0005	0.00098	0.00087
% RSD	0.51	1.80	1.84	1.26

5.4.2.3 Intermediate Precision (Reproducibility)

The RSD values of ETO for interday (0.54-1.09 % and 1.2-1.92 %) and intraday (0.19-0.71 % and 0.37-1.32 %) at 250-260 and 285-295 nm, respectively and the RSD values of SER for interday (1.24-1.90 % and 0.97-1.85 %) and intraday (0.87-1.37 % and 0.55-1.36 %) at 250-260 nm and 285-295 nm reveal that the method is precise.

5.4.2.4 LOD and LOQ

LOD and LOQ values for ETO were found to be 0.39 and 1.18 µg/ml, 0.55 and 1.66 µg/ml at 250-260 and 285-295 nm, respectively. Where, LOD and LOQ values for LOR were found to be 0.59 and 1.80 µg/ml, 0.59 and 1.78 µg/ml at 285-295 nm and 250-260 nm, respectively. Low values of LOD & LOQ indicate that the method is sensitive (Table 5.1).

5.4.2.5 Accuracy (% Recovery)

The recovery experiments were performed by the standard addition method. The mean recoveries were found to be 99.82 ± 0.41 and 100.2 ± 0.93 for ETO and SER respectively. The recoveries results indicate that the proposed method is accurate. Results of recovery studies are shown in Table 5.3.

Table 5.3: Recovery data for the proposed method (n = 3)

Drug	Amount taken (µg/ml)	Amount added (%)	% Recovery ± S. D. (n = 3)
ETO	10	75	101.3 ± 0.51
	10	100	99.32 ± 0.28
	10	125	98.81 ± 0.44
SER	8	75	99.18 ± 1.00
	8	100	100.5 ± 1.00
	8	125	100.9 ± 0.77

S. D. is Standard deviation and n is number of replicates

5.4.3 Assay

The proposed validated method was successfully applied to determine ETO and SER in tablet dosage. Results are given in Table 5.4. No interference of the excipients with the absorbance of analyte of interest appeared; hence the proposed method is suitable for the routine analysis of ETO and SER in tablet dosage.

Table 5.4: Analysis of ETO and SER in tablet dosage by proposed method (n = 6)

Synthetic mixture	Label claim (mg)		Amount found (mg)		% Label claim (mg) (n = 6)	
	ETO	SER	ETO	SER	ETO	SER
1	50	4	50.64	3.93	101.2	98.27
2	50	4	49.42	3.96	98.84	99.12
3	50	4	49.68	4.03	99.36	100.9
4	50	4	49.93	4.10	99.87	102.6
5	50	4	50.06	3.89	100.1	97.41
6	50	4	50.31	4.03	100.6	100.9
Mean			50.01	3.99	100.0	99.85
S.D.			0.44	0.08	0.87	1.92

5.5 CONCLUSION

Based on the results obtained from the analysis using proposed method, it can be concluded that the method has linear response in the range of 2-26 µg/ml for both ETO and SER.

The result of the analysis of tablet dosage by the proposed method is highly reproducible and reliable and is in good agreement with label claim of the drugs. The additive present in the tablet dosage did not interfere in the analysis. So the method can be used for the routine analysis of drugs in combination.

6. DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF ETORICOXIB AND SERRATIOPEPTIDASE

6.1EXPERIMENTAL

6.1.1 Apparatus

- RP-HPLC instrument equipped with a UV-Visible detector and a photodiode array detector, (Shimadzu, LC-2010CHT, Japan,), autosampler, phenomenex C18 column (250 x 4.6 mm, 5 µ particle size) column and LC-solution software
- Analytical balance (Sartorius CP224S, Germany)
- Digital pH meter (LI 712 pH analyzer, Elico Ltd., Ahmedabad)
- Corning volumetric flasks (10, 50, 100 ml)
- Ultra sonic cleaner (Frontline FS 4, Mumbai, India)

6.1.2 Materials and Reagents

- Etoricoxib (ETO) bulk powder was kindly gifted by Sun Pharmaceuticals Ltd., Vadodara, Gujarat, India and Serratiopeptidase (SER) bulk powder was kindly gifted by Acme Pharmaceuticals Ltd., Mehsana, Gujarat, India, respectively
- HPLC grade methanol, acetonitrile, water (Finar Chemicals Ltd., Mumbai, India)
- Ortho-phosphoric acid (Merck Specialties Pvt. Ltd, Worli, Mumbai)
- Nylon 0.45 µm – 47 mm membrane filter (Gelman Laboratory, Mumbai, India)
- Whatman filter paper no. 41. (Whatman International Ltd., England)

6.1.3 Preparation of Solutions & Reagents

6.1.3.1 Preparation of Mobile Phase

Mobile phase (pH 3.0) was prepared by mixing AR grade methanol, acetonitrile and water in the ratio of 60: 30: 10 and the pH adjusted to 3.0 by dilute ortho-phosphoric acid.

6.1.3.2 Preparation of Stock Solutions of ETO (200 µg/ml) and SER (100 µg/ml)

An accurately weighed standard ETO powder (20mg) and SER powder (10 mg) were weighed and transferred to 100 ml separate volumetric flasks and dissolved in mobile phase with sonicator. The flasks were shaken and volumes were made up to mark with mobile phase to give a solution containing 200 µg/ml of ETO and 100 µg/ml of SER.

6.1.3.3 Preparation of Working Standard Solutions

The working standard solutions of ETO and SER were prepared by accurately transferring (0.5, 1, 1.5, 2.5, 3.5 and 5 ml) aliquots of ETO and (0.2, 0.5, 0.8, 1, 1.5 and 2 ml) aliquots of SER to 10 ml volumetric flasks and were made up to mark with mobile phase.

6.1.3.4 Preparation of Sample Solution

ETO (50 mg) and SER (4 mg) standard drug powders were accurately weighed and then mixed with commonly used formulation excipients like starch, lactose, magnesium stearate and talc in appropriate proportion. The mixture was then transferred to 100 ml volumetric flask containing 80 ml mobile phase and sonicated for 30 min. The solution was filtered through Whatman filter paper No. 41 and the volume was adjusted up to the mark with mobile phase. The above solution (1.5 ml) was transferred to 10 ml volumetric flask and diluted up to mark with mobile phase to obtain final concentration of 75 µg/ml ETO and 6 µg/ml SER.

6.1.4 Chromatographic Conditions

- Stationary phase:-Phenomenex C18 (250mm x 4.6mm, i.d-5µm particle size) column at 40ºC temperature.
- Mobile Phase:- methanol: ACN: water (60: 30: 10, v/v/v) (pH-3, adjusted with OPA)
- Flow rate:- 1.0 ml/min
- Injection volume:- 20 µL
- Detection:- The elution was monitored at 255 nm using PDA detector.

6.1.5 Determination of Analytical Wavelength

The standard solutions of ETO and SER were injected under the chromatographic conditions above. Detection was carried out at different wavelength and wavelength at which best response achieved was determined.

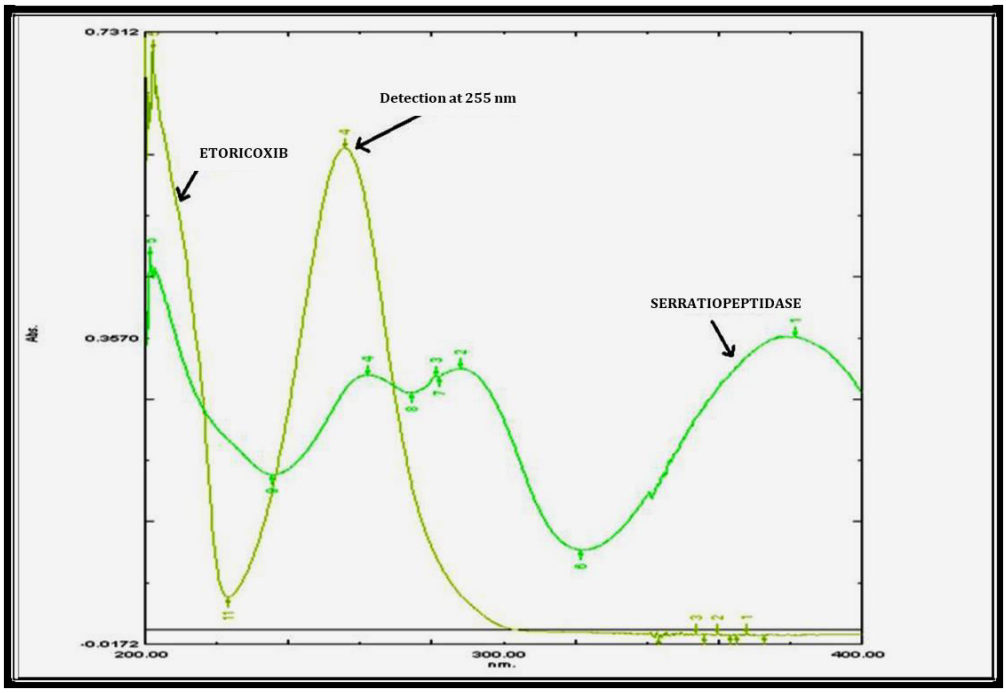


Figure 6.1: Overlain UV Absorption Spectra of standard solutions of ETO (10 µg/ml) and SER (10 µg/ml) in methanol

6.2METHOD VALIDATION

6.2.1 Linearity

Calibration curves were constructed by plotting peak area vs. concentration of ETO and SER, and the regression equations were calculated. The calibration curves were plotted over the concentration range 10-100 µg/ml for ETO and 2-20 µg/ml for SER. From standard stock solutions of ETO (0.5, 1, 1.5, 2.5, 3.5, 5 ml) and of SER (0.2, 0.5, 0.8, 1, 1.5 and 2 ml) were transferred to a series of 10 ml volumetric flasks and diluted to the mark with mobile phase methanol: ACN: water (60: 30: 10, v/v/v) (pH-3, adjusted with OPA). 20 µL of each solution were injected under the operating chromatographic conditions described above.

6.2.2 Method Precision (% Repeatability)

The precision of the instrument was checked by repeated injected six sample solutions of ETO (30µg/ml) and SER (10µg/ml) under the same chromatographic conditions and measurement of peak area, retention time and tailing factor. The low %RSD values (less than 2%) indicate that proposed method is repeatable.

6.2.3 Intermediate Precision (Reproducibility)

The intraday and interday precision of the proposed method was determined by analyzing the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of standard solutions of ETO (20, 30, 50 µg/ml) and SER (5, 10, 15 µg/ml). The result was reported in terms of relative standard deviation (% RSD).

6.2.4 Limit of Detection and Limit of Quantification

LOD and LOQ of drug were calculated using the following equations designated by International Conference on Harmonization (ICH) guidelines.

$LOD = 3.3 \times \sigma/S$
$LOQ = 10 \times \sigma/S$

Where, σ = the standard deviation of the response and S = slope of the calibration curve.

6.2.5 Accuracy (% Recovery)

The accuracy of the method was determined by calculating recoveries of ETO and SER by the standard addition method in two steps. Known amounts of standard solutions of ETO (7.5, 10, 12.5 µg/ml) and SER (3.2, 4, 4.8 µg/ml) were added at 80%, 100% and 120% levels to pre-quantified sample solutions of ETO (10 µg/ml) and SER (4 µg/ml). The amounts of ETO and SER were estimated by applying obtained values to the regression equation of the calibration curve. The value of standard deviation indicates that the proposed method is accurate.

6.2.6 Specificity

The specificity of the method was ascertained by analyzing standard drug solutions and sample solutions of ETO and SER. The peak purity of ETO and SER were assessed for standard solutions and sample solutions of both the drugs.

6.3 ANALYSIS OF DRUGS IN TABLET DOSAGE

The response of the sample solutions were measured at 255 nm under the chromatographic conditions mentioned above for the quantification of ETO and SER. The amounts of ETO and SER present in the solution were determined by applying values of peak area to the regression equations of the calibration curves.

6.4RESULTS AND DISCUSSION

6.4.1Method development

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for ETO and SER was obtained with a mobile phase methanol: ACN: water (60: 30: 10, v/v/v) (pH-3, adjusted with OPA) at a flow rate of 1 ml/min to get better reproducibility and repeatability. Quantification was carried out at 255 nm based on peak area. Complete resolution of the peaks with clear baseline was obtained (Figue 6.2). System suitability test parameters for ETO and SER for the proposed method are reported in Table 6.1.

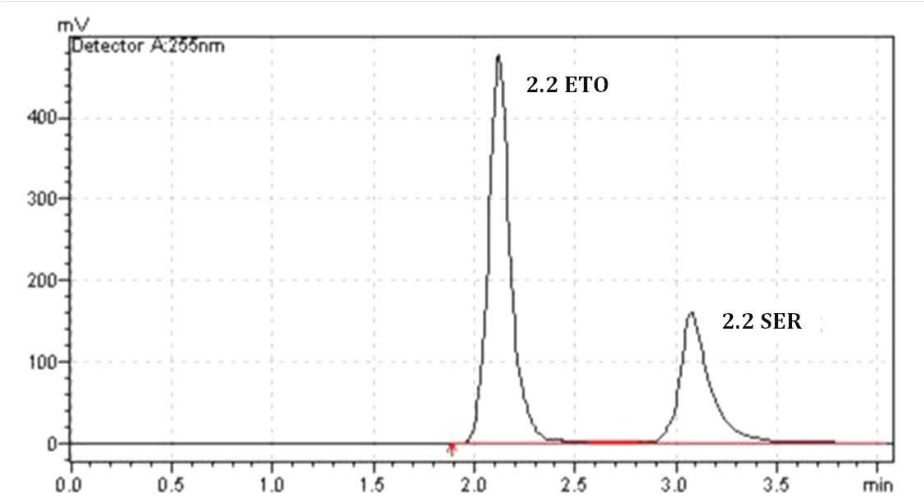


Figure 6.2: Chromatogram of Standard Solution of ETO (50 µg/ml) and SER (50 µg/ml) at 255 nm

Table 6.1: System Suitability Parameters

Parameters	ET ± RSD (n = 6)	SER ± RSD (n = 6)
Retention time (min)	2.20 ± 0.76	3.15 ± 0.48
Tailing factor	1.2 ± 1.62	1.6 ± 1.46
Theoretical plates	2780 ± 0.47	3033 ± 1.12
Resolution	3.16 ± 0.2	

6.4.2 Validation of the proposed method

The proposed method has been validated for the simultaneous determination of ETO and SER in tablet dosage using following parameters.

6.4.2.1 Linearity

Linear correlation was obtained between peak area Vs concentrations of ETO and SER in the concentration range of 10-100 µg/ml for ETO and 2-20 µg/ml for SER. Regression parameters are mentioned in table 7.2 and the calibration curves of these two drugs at 264 nm are shown in Figure 6.3, & Figure 6.4.

Table 6.2: Regression analysis data and summary of validation parameters for the proposed method

PARAMETERS		RP-HPLC method	
		ETO	SER
Detection wavelength(nm)		255	255
Beer s law limit (µg/ml)		10-100	2-20
Regression equation y = mx + c		y = 66969x –15461	y = 35056x + 7922
Slope(m)		66969	35056
Intercept(c)		15461	7922
Correlation coefficient (r^2)		0.9917	0.9982
Repetability (% RSD, n = 6)		0.31	0.59
Precision (%RSD)	Intraday(%RSD)	0.10-0.39	0.55-1.02
	Interday(%RSD)	0.15-0.46	0.61-1.17
LOD(µg/ml)		0.35	0.19
LOQ(µg/ml)		1.06	0.58
Accuracy ± S. D. (% Recovery, n = 3)		99.07 ± 0.42	100.8 ± 1.05

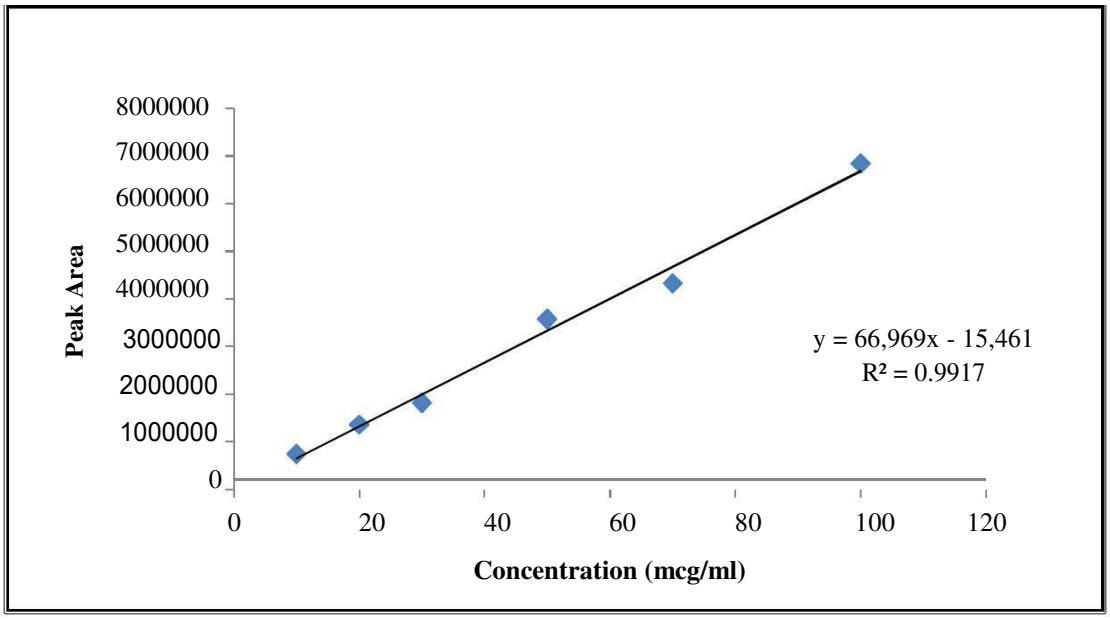


Figure 6.3: Calibration Curve of ETO at 255 nm

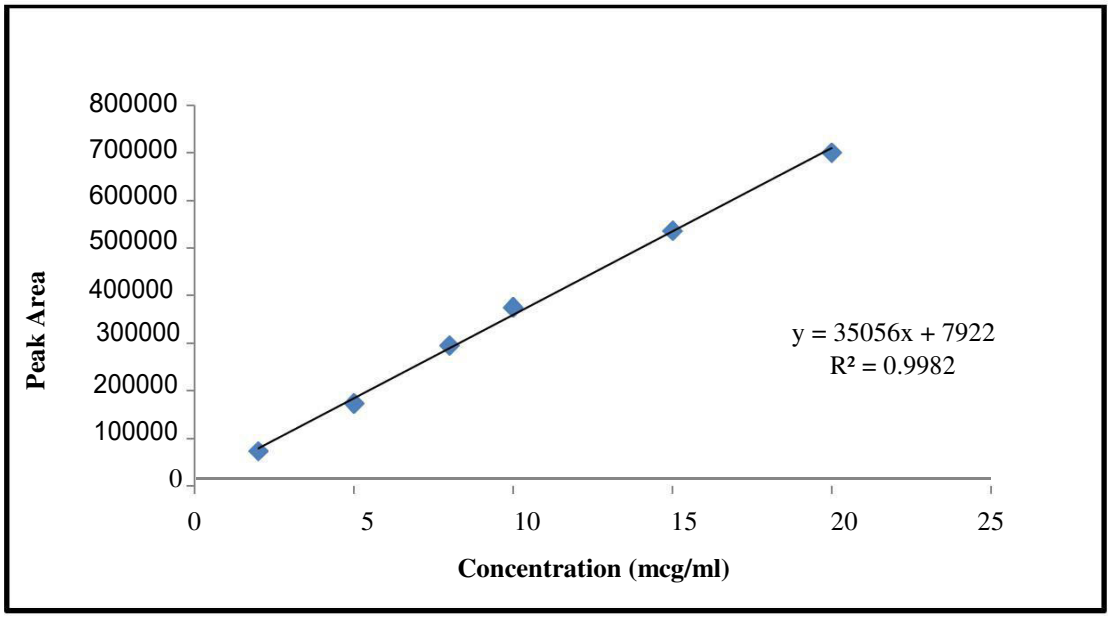


Figure 6.4: Calibration Curve of SER at 255 nm 6.4.2.2

Method Precision (Repeatability)

The RSD values for ETO and SER were found to be 0.31 and 0.59 %, respectively (Table 6.2 & 6.3). The RSD values were found to be < 2 %, which indicates that the proposed method is repeatable.

Table 6.3: Precision Data for ETO and SER

Concentration (ETO: SER)	Retention time (min)		Peak area		Tailing factor	
(30:10 µg /ml)	ETE	SER	ETO	SER	ETO	SER
1	2.21	3.16	1813678	371649	1.20	1.57
2	2.20	3.15	1814434	375939	1.21	1.59
3	2.18	3.13	1817370	376450	1.21	1.61
4	2.22	3.17	1825456	372059	1.18	1.62
5	2.18	3.15	1827458	372569	1.17	1.57
6	2.21	3.17	1820367	375721	1.22	1.62
Mean	2.2	3.15	1819794	374064	1.20	1.60
SD	0.02	0.01	5712	2193	0.02	0.02
% RSD	0.76	0.48	0.31	0.59	1.62	1.46

6.4.2.3 Intermediate Precision (Reproducibility)

The low RSD values of interday (0.15 - 0.46 % and 0.61 – 1.17 %) and intraday (0.10 – 0.39 % and 0.55 - 1.02 %) for ETO and SER, respectively, reveal that the proposed method is precise (Table 6.2).

6.4.2.4 LOD and LOQ

LOD values for ETO and SER were found to be 0.35 µg/ml and 0.19 µg/ml, respectively and LOQ values for ETO and SER were found to be 1.06 µg/ml and 0.58 µg/ml, respectively (Table 6.2). These data show that the proposed method is sensitive for the determination of ETO and SER.

6.4.2.5 Accuracy (% Recovery)

The recovery experiment was performed by the standard addition method. The recoveries obtained were 99.07 ± 0.42 % and 100.8 ± 1.05 % for ETO and SER,

respectively. The low value of standard deviation indicates that the proposed method is accurate. Results of recovery studies are shown in Table 6.4

Table 6.4: Recovery Data for the proposed method

Drug	Level	Amount of sample taken (µg/ml)	Amount of standard spiked (%)	Mean % Recovery ± RSD
ETO	I	10	80 %	98.79 ± 0.73
	II	10	100 %	98.99 ± 0.31
	III	10	120 %	99.42 ± 0.22
SER	I	4	80 %	99.79 ± 1.46
	II	4	100 %	101.6 ± 1.02
	III	4	120 %	101.0 ± 0.65

6.4.2.6 Specificity

The specificity of the method was ascertained by analyzing standard solutions and for sample ETO and SER. The peak purity of std. ETE and SER were 1.000 and 0.9999 respectively, and for the sample ETO and SER peak purity were 0.9998 and 0.9998. The above results suggest that proposed method is specific for the simultaneous estimation of ETO and SER.

Table 6.5: Peak purity index of standard and sample solution of ETO and SER

Sr No	Preparation	Peak purity	
		ETO	SER
1	Standard solution	1.0000	0.9999
2	Sample solution	0.9998	0.9998

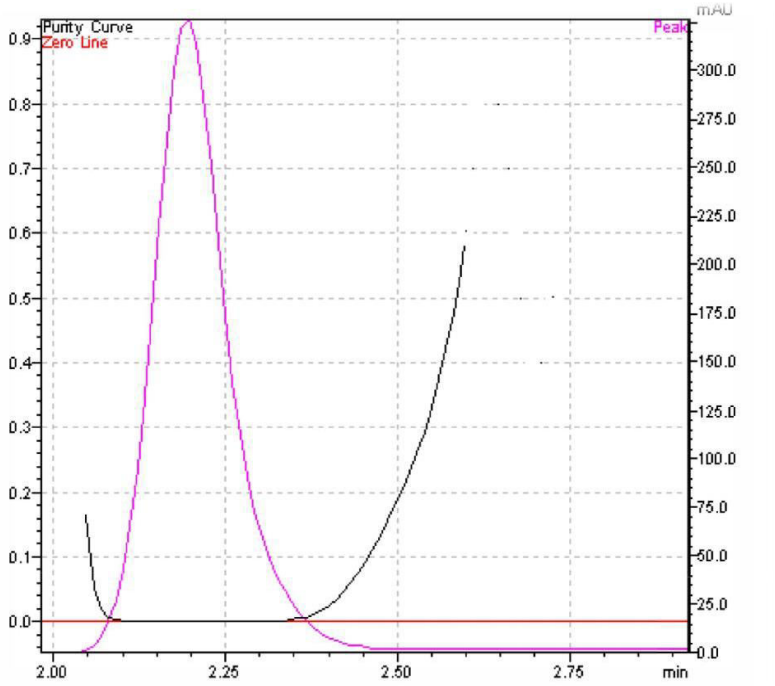


Figure 6.5 Peak Purity index of standard solution of ETO (50 µg/ml)

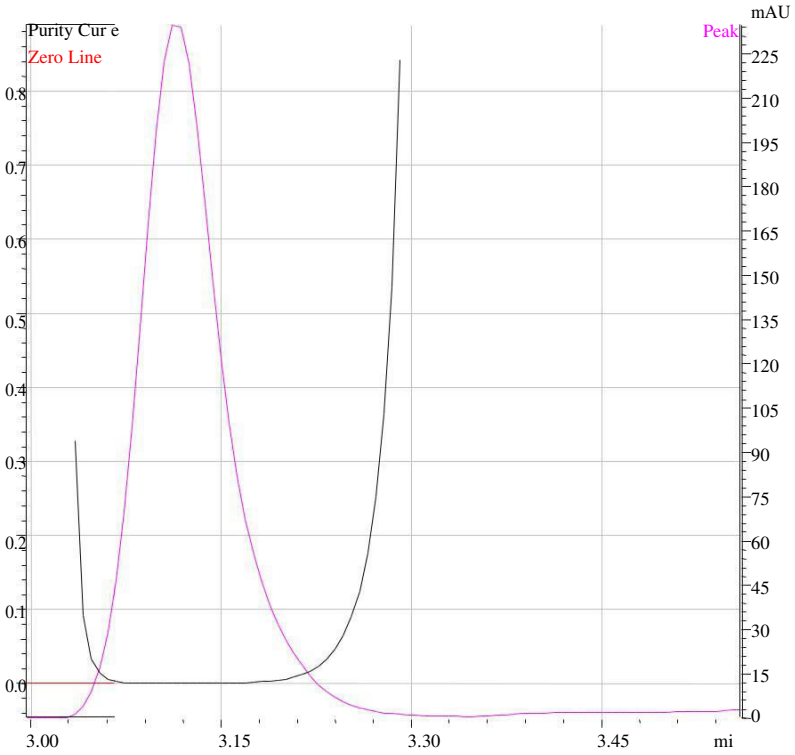


Figure 6.6 Peak Purity index of standard solution of SER (50 µg/ml)

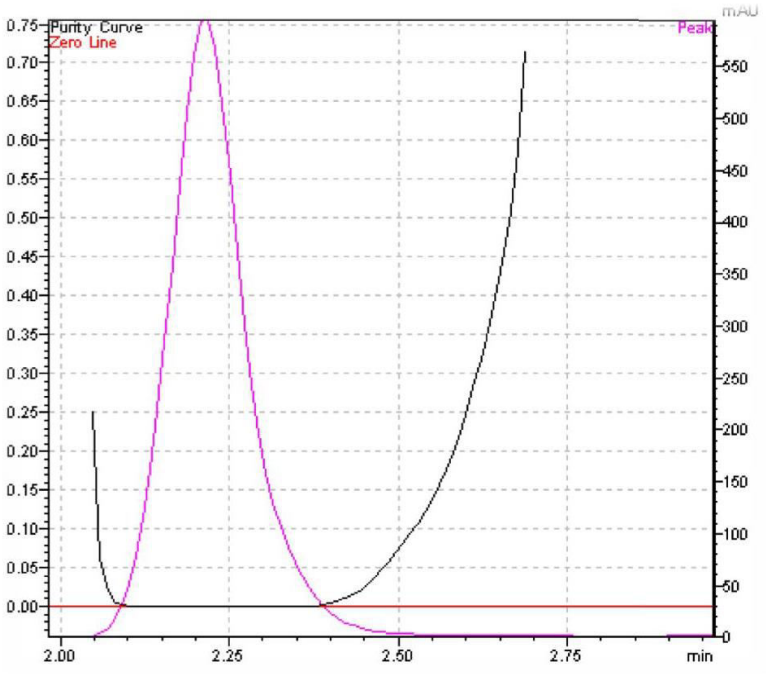


Figure 6.7 Peak Purity index of sample solution of ETO (75 µg/ml)

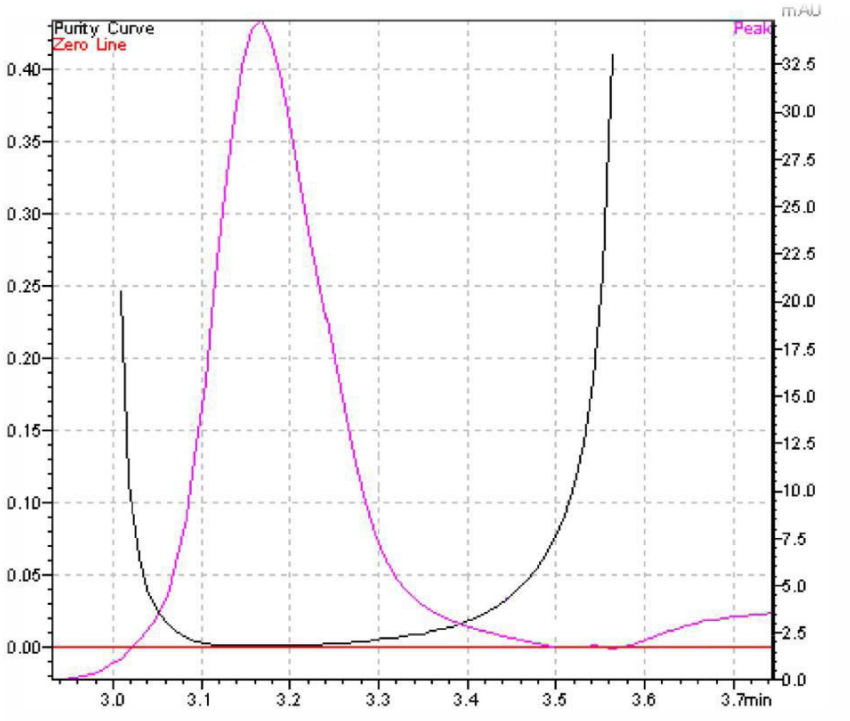


Figure 6.8 Peak Purity index of sample solution of SER (6 µg/ml)

6.4.3 Assay of the tablet dosage

The proposed validated method was successfully applied to determine ETO and SER in their tablet dosage. The result obtained for ETO and SER was comparable with the corresponding labeled amounts (Table 7.6). The RP-HPLC chromatogram for ETO and SER in sample was recorded and is shown in Figure 6.5.

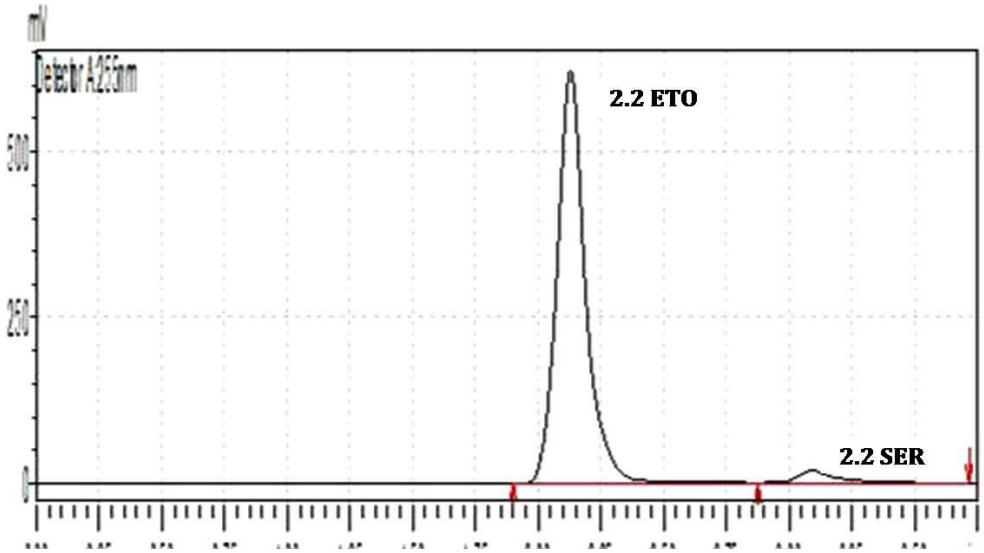


Figure 6.9: Chromatogram of sample solution of ETO (75 µg/ml) and SER (6 µg/ml) at 255 nm

Table 6.6: Analysis of tablet dosage of ETO and SER by proposed method (n = 6)

Sample No.	Label Claim		Amount Found		% Label Claim	
	ETO (mg)	SER (mg)	ETO (mg)	SER (mg)	ETO (%)	SER (%)
1	50	4	49.19	4.01	98.39	100.18
2	50	4	49.79	3.97	99.58	99.39
3	50	4	49.58	4.03	99.17	100.9
4	50	4	50.14	4.06	100.3	101.6
5	50	4	49.49	3.99	98.99	99.88
6	50	4	50.78	4.07	1012	101.8
Mean			49.83	4.02	99.67	100.6
S.D.			0.56	0.04	1.12	0.96

6.5 CONCLUSION

In this proposed method, the linearity is observed in the concentration range of 10-100 µg/ml and 2-20 µg/ml with co-efficient of correlation, (r^2) = 0.991 and (r^2) = 0.998 for ETO and SER, respectively at 255 nm. The results of the analysis of tablet dosage by the proposed method are highly reproducible and reliable and it is in good agreement with the label claim of the drug. The method can be used for the routine analysis of the ETO and SER in combination without any interference of excipients.

7. COMPARISON OF ASSAY RESULTS OF THE DEVELOPED AND VALIDATED ANALYTICAL METHODS

7.1 ANALYSIS OF VARIANCE TEST (ANOVA) [41-42]

7.1.1 Definition

ANOVA is the “separation of variance ascribable to one group of causes from the variance ascribable to another group.”

7.1.2 Use of ANOVA

For designs with one independent variable with more than 2 groups or levels, using inferential statistics to determine if there are differences between these groups – we use Analyses of variance (ANOVA) to analyze data. ANOVA uses the F-statistic or F-ratio to determine if differences between groups are significant.

7.1.3 Techniques

The analysis of variance technique can be applied as follows:

- One way classification means we consider the influence of only one factor.
- Two way classification means we consider the influence of two factors at

a time. In ANOVA require to calculate following terms:

a) Correction factor C.F. = T^2/N, where T is grand total.

b) Sums of squares of variations amongst the C.S.S. (column sums of squares)

C.S.S. = $C1^2/n1 + C2^2/n2 + \ldots$ -

C.F.Where,C1,C2…etc. are

column totals.

n1, n2 … etc. are numbers of observations in respective columns.

c) Total sums of squares (T.S.S.)

T.S.S. = (sum of squares of each observation) – C.F.

d) Sums of squares of variations within or error

(E.S.S.) E.S.S. = T.S.S. – (C.S.S.)

Table 7.1: ANOVA

Source	Degrees of freedom	Sum of squares	Mean sum of squares	'F' ratio
Between the columns (C.S.S.)	c - 1	C.S.S.	C.S.S. / c - 1	Large value / Smaller value
Error	Difference between T.S.S. and C.S.S		S.S. / N -c	
Total	N - 1	T.S.S.	-	-

Note: While calculating F ratio we compare mean sum of squares for

(i) Rows and error.

(ii) Columns and error and calculate F ratio as

$$F = \frac{\text{Large value}}{\text{Smaller value}}$$

7.2 ANOVA FOR THE DEVELOPED AND VALIDATED METHODS FOR ETORICOXIB AND SERRATIOPEPTIDAS

For the comparison of the four developed methods i.e. simultaneous equations method, first order derivative method, AUC method, RP-HPLC method for the simultaneous estimation of Etoricoxib and Serratiopeptidase in tablet dosage - use analyses of variance (ANOVA).

In the ANOVA test we have to test null hypothesis (H0) against the alternative hypothesis (H1).Let H0 = There are no significant differences among the four methods.

H1 = There are significant differences among the four methods.

Table 7.2: ANOVA results ofEtoricoxib

Sr. No.	Simultaneous equations method	First derivative method	Area under curve method	RP-HPLC Method
1	101.88	98.97	101.2	98.39
2	102.04	98.81	98.84	99.58
3	102	100.35	99.36	99.17
4	102.16	100.8	99.87	100.29
5	97.48	99.4	100.12	98.99
6	102.08	99.46	100.6	101.56
Mean	101.27	99.63	99.99	99.66
F - value	2.36 (< 3.10)			

Table 7.3: ANOVA calculations for Etoricoxib

Source of variation	Sum of squares (SS)	Degree of freedom (d. f.)	Mean squares (MS)	Variance ratio F
Between the methods	10.74	3	3.58	F_c = 3.58/1.51 = 2.36
Within the methods (error)	30.28	20	1.51	
Total	41.02953	23	-	

The calculated value of F is 2.36

Here $F_{calculated} = 2.36 < F_{tabulated} = 3.10$ (P = 0.05).

So, it can be concluded that there is no significant difference among the above four methods. Also there is no significant difference observed to developed method by analysis.

Table 7.4: ANOVA results for Serratiopeptidase

Sr. No.	Simultaneous equations method	First derivative method	Area under curve method	RP-HPLC Method
1	102.5	98.48	98.27	100.18
2	101.5	100	99.12	99.39
3	102.5	101.51	100.9	100.89
4	101	102.27	102.6	101.56
5	98.5	101.51	97.41	99.88
6	102.15	103.03	100.9	101.81
Mean	101.36	101.13	99.87	100.62
F - value	1.08 (< 3.10)			

Table 7.5: ANOVA calculations for Serratiopeptidase

Source of variation	Sum of squares (SS)	Degree of freedom (d. f.)	Mean squares (MS)	Variance ratio F
Between the methods	7.89	3	2.63	F_c = 2.63/2.42 = 1.08
Within the methods (error)	48.42	20	2.42	
Total	56.31	23	-	

The calculated value of F is 1.08

Here $F_{calculated} = 1.08 < F_{tabulated} = 3.10$ (P = 0.05).

So, it can be concluded that there is no significant difference among the above four methods

SUMMARY AND CONCLUSION

8. SUMMARY

SUMMARY OF ALL METHODS

- Spectroscopic methods like Simultaneous equations method, First order derivative method and Area under curve method were developed for the simultaneous estimation of Etoricoxib and Serratiopeptidase in tablet dosage.
- The developed spectroscopic methods were validated for linearity, accuracy, method precision, intra-day and inter-day precision, limit of detection and limit of quantification.
- RP-HPLC method was developed for the simultaneous estimation of Etoricoxib and Serratiopeptidase in tablet dosage.
- The developed RP-HPLC method was validated for linearity, accuracy, method precision, intra-day and inter-day precision, limit of detection, limit of quantification.
- All the validated analytical methods were compared by applying some statistical treatment like ANOVA.
- All the developed and validated methods can be successfully applied to determine the drugs in the tablet dosage.

Table 8.1: Summary of validation parameters for Simultaneous equations method and First order derivative method

Parameters	Simultaneous equations method				First order derivative method	
Wavelength (nm)	**ETO 255**	**ETO 288**	**SER 288**	**SER 255**	**ETO 264**	**SER 225.2**
Range (µg/ml)	2-30	2-30	2-30	2-30	2 - 30	2 - 14
Correlation Coefficient (r^2)	0.9998	0.9956	0.9957	0.9954	0.9984	0.9956
% Recovery (n = 3)	99.5 ± 0.26		99.87 ± 0.35		98.87 ± 0.36	100.4 ± 0.69
Repeatability (% RSD, n = 6)	0.34	0.54	0.17	0.20	1.12	1.29
Interday (% RSD, n = 3)	0.21-0.49	0.42-0.95	0.13-0.32	0.23-0.47	0.29 – 1.15	0.97 – 1.33
Intraday (% RSD, n = 3)	0.16-0.34	0.40-0.72	0.12-0.24	0.11-0.33	0.36 – 0.56	0.68 – 1.12
LOD (µg/ml)	0.36	0.43	0.26	0.24	0.36	0.23
LOQ (µg/ml)	1.10	1.29	0.79	0.72	1.09	0.71
Sandell's sensitivity (µg/cm^2/0.001 absorbance unit)	0.01725	0.1449	0.03299	0.03640	-----	-----
Molar extinction co-efficient (l mol^{-1} cm^{-1})	17145.69	20413.45	11270.60	10213.52	-----	-----
Assay ± SD	101.3 ± 1.86		101.4 ± 1.52		99.63 ± 0.78	101.1 ± 1.64

Table 8.2: Summary of validation parameters for Area under curve method and RP-HPLC method

Parameters	Area under curve method				RP-HPLC	
Wavelength (nm)	**ETO 250-260**	**ETO 285-295**	**SER 285-295**	**SER 250-260**	**ETO 255**	**SER 255**
Range (µg/ml)	2-26	2-26	2-26	2-26	10-100	2-20
Correlation Coefficient (r^2)	0.9998	0.9976	0.9977	0.9964	0.9917	0.9982
% Recovery (n = 3)	99.82 ± 0.41		100.2 ± 0.93		99.07 ± 0.42	100.8 ± 1.05
Repeatability (% RSD, n = 6)	0.51	1.80	1.26	1.85	0.31	0.59
Interday (% RSD, n = 3)	0.54-1.09	1.2-1.92	0.97-1.85	1.24-1.90	0.15-0.46	0.61-1.17
Intraday (% RSD, n = 3)	0.19-0.71	0.37-1.32	0.55-1.36	0.87-1.37	0.10-0.39	0.55-1.02
LOD (µg/ml)	0.39	0.55	0.59	0.59	0.35	0.19
LOQ (µg/ml)	1.18	1.66	1.80	1.78	1.06	0.58
Assay ± SD	100.0 ± 0.87		99.85 ± 1.92		99.67 ± 1.12	100.6 ± 0.96

From the results, we can conclude that all methods are precise, linear, accurate and repeatable and so they can be applied for the estimation Etoricoxib and Serratiopeptidase in tablet dosage. The RP-HPLC method is more sensitive than other methods and it is also more precise, repeatable and specific.

BIBLIOGRAPHY

9.BIBLIOGRAPHY

1. Kathleen G. Pharmacotherapeutics: Clinical reasoning in primary cares, 2nd edition, Churchill livingstone: Sounders Elsevier; 2003, p.374-388.
2. Rang HP, Dale MM, Ritter JM, Flower RJ. Pharmacology, 6th edition, Churchill livingstone: Elsevier; 2007, p.584-587.
3. Barar,F.S.K.Essentialof Pharmacotheraputics,3rd Edition;2000,p.221-227.
4. Sweetman SC. Martindale: The Complete Drug Reference, 35th edition, London: Pharmaceutical Press; 2007, p.1736
5. Maryadele. J. O' Neil. The Merck Index: An Encyclopedia of chemicals, drugs and biologicals, 13th edition, New Jersey: Published by Merck Research Laboratories, Division of Merck and Co., Inc. Whitehouse station, p. 615
6. Sweetman SC. Martindale: The Complete Drug Reference, 35th edition, London: Pharmaceutical Press; 2007, p.66
7. Maryadele. J. O' Neil. The Merck Index: An Encyclopedia of chemicals, drugs and biologicals, 13th edition, New Jersey: Published by Merck Research Laboratories, Division of Merck and Co., Inc. Whitehouse station; 2006, p. 967
8. ICH Harmonized Tripartile Guideline (2005). Validation of analytical procedures: Text and Methodology Q2(R1), International Conference on Harmonization, Geneva, Switzerland
9. Beckett AH, Stenlake JB. Practical pharmaceutical chemistry, 14th edition, CBS publishers and distributors: New Delhi; 1997, p.275-337.
10. Sharma BK, Instrumental methods of chemical analysis, 20th edition, Goel publishing house: 2001; p.54-83.
11. Mendham J, Denney RC, Barners JD, Thomas MJK. Vogel's textbook of quantitative chemical analysis. 6th edition, Pearson education: London; 2006, p. 217-235.
12. Skoog DA, Holler FJ, Nieman TA. Principle of instrumental analysis. 2nd edition, Thomson books/cole: Philadelphia; 1980, p. 690-698.
13. Gangane P.S, Bagde SM, Mujbaile SG, Niranjane KD, (2014). Development and Validation of HPLC assay method for Etoricoxib in bulk drug and tablet formulation. Indian journal of Natural sciences. 4(24): 1565-72.

14. Adidala RR, Rajamani K, Mounika A, (2015). Development and validation of RP-HPLC method for simultaneous estimation of Paracetamol, Aceclofenac and Serratiopeptidase in combined tablet dosage form. World journal of Pharmacy and Pharmaceutical Sciences. 4(3): 1008-23.
15. Syed Mujtaba Ahmed, L. PriyadarsiniRaparla, Mohammed Omer, (2015). RP-HPLC method development and validation for simultaneous estimation of diclofenac sodium and serratiopeptidase in tablet dosage form. International Journal of Research in Pharmaceutical and Nano Sciences. 4(1):10-18
16. Ashish Sharma D, PrajapatiLaxman M, Joshi Amit K, KharodiyaMahammadali L. Rajpurohit Prashant T, Patel Dhaval, RamaniSandip, (2014). UV spectrophotometric method for the simultaneous estimation of Serratiopeptidase and Nimesulidein their bulk and combined dosage form.International Journal of Universal Pharmacy and Bio Sciences. 3(3): 157-74.
17. Ashok R. Parmar, Dharmishtha N, Bhakhar, Dolita K, and Kinjal VV, (2012). Simultaneous estimation of Aceclofenac and Serratiopeptidase in Tablet dosage form by absorbance ratio method using visible spectrophotometry. Der Pharmacia Sinica. 3(3):321-326.
18. Vaishali DR, Kapse SN, Sadhana Mahajan, (2014). Simultaneous Estimation of Serratiopeptidase and Diclofenac Sodium by UV-Spectrophotometric Method. Journal of Current Pharma Research.4(4): 1297-1301.
19. Vishnu P. Choudhari, Sanket R. Parekar, Subhash G. Chate, Pradeep D. Bharande, Bhanudas S. Kuchekar (2011). Development and validation of UV-visible spectrophotometric baseline manipulation methodology for simultaneous analysis of drotraverine and etoricoxib in pharmaceutical dosage forms. Pharmaceutical methods. 2(4): 247-252.
20. Krishnaveni G, Sathyannaryana PVV, (2012). A Novel RP-HPLC method for the Quantification of Celecoxib in Formulations.Research Journal of Pharmaceutical, Biological and Chemical Sciences. 3(1): 340-346.
21. Chaple DR, Fandi DS, Chaple PD, (2010). Simultaneous Estimation of Etoricoxib and Paracetamol in Combined Tablet Dosage Form. Research Journal of Pharmaceutical, Biological and Chemical Sciences. 1(3): 485-89.

22. Suresh Kumar S, Natraj D, Asadulla Khan, Kalyan Kumar B, Venkateswara Rao J, (2011). Development and Validation of RP-HPLC Method for Simultaneous estimation of Etoricoxib and Thiocolchicoside in Pharmaceutical Dosage Forms. International Journal of Research in Pharmacy and Chemistry. 1(3): 649-653.
23. Krishna Gupta R, AmrutaLikhar and Sudhir G Wadodkar, (2010). Application of Stability Indicating HPLC Method for Quantitative Determination of Etoricoxib and Paracetamol in Pharmaceutical Dosage Form. Eurasian J. Anal. Chem. 5(3): 218-26
24. Sanjiv Kumar, Amit Joshi, Rahul Singh Thakur, Anupam K. Pathak and Kamal Shah, (2011). Simultaneous Estimation of EtoricoxibAndThiocolchicoside by RP-HPLC Method in Combined Dosage Forms. ActaPoloniaePharmaceutica. 68(6): 839-845.
25. SachinGholve, OmprakashBhusnure, Oommen Mathew, JaiprakashSangshetti, (2016). Development and Validation of A RP - HPLC Method For Determination of Etoricoxib in Pharmaceutical Dosage Forms.Int J Pharm Bio Sci. 7(2): 246 – 53.
26. Venkatachalam ,lalitha KG, (2013).Reverse – phase HPLC method for simultaneous analysis of Thiocolchicoside and Ketoprofen in tablet formulations. International Journal of Pharmacy and Pharmaceutical Sciences. 5(4): 679-82.
27. Vaishalia, Vikas KS, (2016). Development and validation of RP-HPLC method for the assay of Celecoxib capsule. International Journal of Pharmaceutical Sciences. 2(1): 22-6.
28. Prinesh N. Patel, GananadhamuSamanthula, VishalkumarShrigod, Sudipkumar C. Modh, and Jainishkumar R. Chaudhari, (2013). RP-HPLC Method for Determination of Several NSAIDs and Their Combination Drugs. Chromatography Research International. 3(1): 24-37.
29. Gajera JB, Hasumati AR, Vineet CJ, (2014). Simultaneous Estimation of Tizanidine Hydrochloride and Rofecoxib by Ratio Derivative Spectroscopic Method in Tablet. Pharma Science Monitor 5(2): 141-52.
30. Sekar V, Jayaseelan S, Udhaya Kumar E, Subash N, Prakash M, Perumal P, (2010). Development and Validation Of RP-HPLC Method for the simultaneous estimation of Diacerein and Aceclofenac In Dosage Form. International Journal of ChemTech Research. 2(1): 168-71

31. Raju Chandra, AshwaniSanghi, Deepak Kumar and Krishna Kumar , (2014). Develop a Simple RP-HPLC and UV-Visible Method for estimation of Etoricoxib from Pharmaceutical Dosage. British Biomedical Bulletin. 2(4): 703-713.
32. Syal PK, Sahoo M, Ingale KD, Ingale SS, Choudhari VP, Kuchekar BS, (2010). Development and validation of a RP-HPLC-PDA method for simultaneous estimation of Drotaverine and Etoricoxib in tablet and its application for dissolution studies. Der Pharma Chemica, 2010, 2(4): 93-102.
33. Manish Kumar Thimmaraju, Venkat Rao, Hemanth, Siddartha P, (2011). RP HPLC method for the determination of Etoricoxib in bulk and pharmaceutical formulations. Der Pharmacia Lettre, 2011: 3 (5) 224-231.
34. Ashok R. Parmar, Dharmishtha N. Bhakhar, Dolita K. Shah, Kinjal V. Vekariya, (2012). Simultaneous estimation of Aceclofenac and Serratiopeptidase in Tablet Dosage Form by Absorbance Ratio Method using Visible Spectrophotometry. Der Pharmacia Sinica. 3 (3):321-326
35. Aayushi, Agarwal Bansal (2014). Development and Validation of an Analytical Method for the Estimation of Etoricoxib Tablets by Reverse Phase HPLC. J. Pharm. Res. 2014, 3(1), 1 – 6.
36. VijayaKuchana, ShruthiPolupongu, Naveen Kumar CH, Sunitha P, (2014). A New Validated RP-HPLC Method for Estimation of Etoricoxib in its Bulk and Pharmaceutical Dosage forms. Journal of Scientific Research in Pharmacy. 3(4): 124-127.
37. KalpanaNekkala, V. Shanmukha Kumar J, Ramachandran D, GanjiRamanaiah, (2015). Development and validation of stability indicating RP-LC method for estimation of celecoxib (CXB) in microemulsion capsule formulations. Journal of Chemical and Pharmaceutical Research. 7(7):766-774.
38. Pattan SR, Jamdar SG, Godge RK, Dighe NS, Daithankar AV, Nirmal SA. (2009). RP- HPLC Method for Simultaneous Estimation of Paracetamol and Etoricoxib from Bulk and Tablets. Journal of Chemical and Pharmaceutical Research. 1(1): 329-335.

39. Santosh R. Karajgi, SomashekharMetri, Vivek Tiwari, SujayHulyalkar, Tanveer Abdul Rub and Amruta S. Patil, (2016). UV spectrophotometric method for the quantitative estimation of celecoxib in capsule dosage forms . Der Pharmacia Lettre, 8 (10):247-257.
40. Revathi, R. VenkateshaPerumal, S. Sudharshini, A.MohammedAnsar, A. Thilagalakshmi, and A. P. Dinesh, (2011). Simple UV Spectrophotometric Determination of Celecoxib in Pure Form and in Pharmaceutical Formulations. International Journal of Pharmaceutical Sciences Letters. 1(2): 49-50.
41. Patel GC, Jani GK. Basic biostatistics for pharmacy, 1st edition, Ahmedabad: Atulprakashan; 2006, p.183-185.
42. Shah YI, Paradkar AR, Dhayagude MG. Introduction to biostatics and computer science, 7th edition, Pune: Niraliprakashan; 2006, p. 7.1-7.2.